



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/68, C07H 15/12, 17/00		A1	(11) International Publication Number: WO 92/17484 (43) International Publication Date: 15 October 1992 (15.10.92)
<p>(21) International Application Number: PCT/US92/02480</p> <p>(22) International Filing Date: 26 March 1992 (26.03.92)</p> <p>(30) Priority data: 675,843 27 March 1991 (27.03.91) US</p> <p>(71) Applicant: RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 6840 East Broadway Boulevard, Tucson, AZ 85710 (US).</p> <p>(72) Inventor: KOOL, Eric, T. ; 39 Brighton Street, Apt. 1, Rochester, NY 14600 (US).</p> <p>(74) Agent: SCOTT, Anthony, C.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: SINGLE-STRANDED CIRCULAR OLIGONUCLEOTIDES</p> <p>(57) Abstract</p> <p>The present invention provides single-stranded circular oligonucleotides each with a parallel binding (P) domain and an anti-parallel binding (AP) domain separated from each other by loop domains. Each P and AP domain has sufficient complementarity to bind to one strand of a defined nucleic acid target wherein the P domain binds in a parallel manner to the target and the AP domain binds in an anti-parallel manner to the target. Moreover, the present single-stranded circular oligonucleotides can bind to both single-stranded and double-stranded target nucleic acids. The present invention also provides methods of using these oligonucleotides as well as pharmaceutical compositions containing these oligonucleotides.</p>			

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/02480

A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) : C12Q 1/68; C07H 15/12, 17/00
US CL : 435/6, 536/27, 28, 29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US. A. 4,766,062 (Diamond et al) 23 August 1988, see entire document.	1-64

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document number of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 July 1992

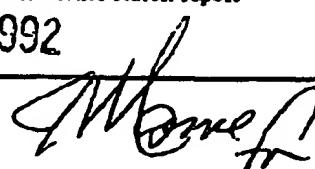
Date of mailing of the international search report

28 JUL 1992

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

L. YUAN



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

-1-

1

SINGLE-STRANDED CIRCULAR OLIGONUCLEOTIDES

The present application is a continuation-in-part of copending U.S. Serial No. 675,843 filed March 5 27, 1991. Moreover, the subject matter of the present application relates to subject matter contained in Disclosure Document number 234,794 received by the United States Patent and Trademark Office on September 5, 1989.

10 This invention was made with United States government support under grant number GM-46625 awarded by the National Institutes of Health. The United States government has certain rights in the invention.

15 FIELD OF THE INVENTION:

The present invention provides single-stranded circular oligonucleotides capable of binding to a target DNA or RNA and thereby regulating DNA replication, RNA transcription, protein translation, and other processes 20 involving nucleic acid templates. Furthermore, circular oligonucleotides can be labeled for use as probes to detect or isolate a target nucleic acid. Circular oligonucleotides can also displace one strand of a duplex nucleic acid without prior denaturation of the 25 duplex. Moreover, circular oligonucleotides are ~~resistant to exonucleases~~ and bind to a target with higher selectivity and affinity than do linear oligonucleotides.

30 BACKGROUND OF THE INVENTION:

An oligonucleotide binds to a target nucleic acid by forming hydrogen bonds between bases in the

1 target and the oligonucleotide. Common B DNA has conventional adenine-thymine (A-T) and guanine-cytosine (G-C) Watson and Crick base pairs with two and three hydrogen bonds, respectively. Conventional 5 hybridization technology is based upon the capability of sequence-specific DNA or RNA probes to bind to a target nucleic acid via Watson-Crick hydrogen bonds. However, other types of hydrogen bonding patterns are known wherein some atoms of a base which are not involved in 10 Watson-Crick base pairing can form hydrogen bonds to another nucleotide. For example, thymine (T) can bind to an A-T Watson-Crick base pair via hydrogen bonds to the adenine, thereby forming a T-AT base triad. Hoogsteen (1959, *Acta Crystallography* 12: 822) first 15 described the alternate hydrogen bonds present in T-AT and C-GC base triads. More recently, G-TA base triads, wherein guanine can hydrogen bond with a central thymine, have been observed (Griffin et al., 1989, *Science* 245: 967-971). If an oligonucleotide could bind 20 to a target with both Watson-Crick and alternate hydrogen bonds an extremely stable complex would form that would have a variety of in vivo and in vitro utilities. However, to date there has been no disclosure of an oligonucleotide with the necessary 25 structural features to achieve stable target binding with both Watson-Crick and alternate hydrogen bonds.

Oligonucleotides have been observed to bind by non-Watson-Crick hydrogen bonding in vitro. For example, Cooney et al., 1988, *Science* 241: 456 disclose 30 a 27-base single-stranded oligonucleotide which bound to a double-stranded nucleic acid via non-Watson-Crick hydrogen bonds. However, triple-stranded complexes of

1 this type are not very stable, because the oligonucleotide is bound to its target only with less stable alternate hydrogen bonds, i.e., without any Watson-Crick bonds.

5 Oligonucleotides have been used for a variety of utilities. For example, oligonucleotides can be used as probes for target nucleic acids that are immobilized onto a filter or membrane, or are present in tissues.

10 Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Press, NY) provide a detailed review of hybridization techniques.

Furthermore, there has been great interest recently in developing oligonucleotides as regulators of cellular nucleic acid biological function. This 15 interest arises from observations on naturally occurring complementary, or antisense, RNA used by some cells to control protein expression. However, the development of oligonucleotides for in vivo regulation of biological processes has been hampered by several long-standing 20 problems, including the low binding stability and nuclease sensitivity of linear oligonucleotides.

For example, transcription of the human c-myc gene has been inhibited in a cell free, in vitro assay system by a 27-base linear oligonucleotide designed to 25 bind to the c-myc promoter. Inhibition was only observed using a carefully controlled in vitro assay system wherein lower than physiological temperatures were employed, and many cellular enzymes had been removed or inactivated. These conditions were necessary 30 because linear oligonucleotides bind with low affinity and are highly susceptible to enzymes which degrade linear pieces of DNA (Cooney et al.). Splicing of a

1 pre-mRNA transcript essential for Herpes Simplex virus replication has also been inhibited with a linear oligonucleotide which was complementary to an acceptor splice junction. In this instance, a methylphosphonate 5 linkage was employed in the linear oligonucleotide to increase its nuclease resistance. Addition of this chemically-modified oligonucleotide to the growth medium caused reduction in protein synthesis and growth of uninfected cells, most likely because of toxicity 10 problems at high concentrations (Smith et al., 1986, Proc. Natl. Acad. Sci. USA 83: 2787-2791).

In another example, linear oligonucleotides were used to inhibit human immunodeficiency virus replication in cultured cells. Linear oligonucleotides 15 complementary to sites within or near the terminal repeats of the retrovirus genome and within sites complementary to certain splice junctions were most effective in blocking viral replication. However, these experiments required large amounts of the linear 20 oligonucleotides before an effect was obtained, presumably because of the low binding stability and vulnerability of these linear oligonucleotides to nucleases (Goodchild et al., 1988, Proc. Natl. Acad. Sci. USA 85: 5507-5511).

25 Accordingly, oligonucleotides that are useful as regulators of biological processes preferably possess certain properties. First, the oligonucleotide should bind strongly enough to its complementary target nucleic acid to have the desired regulatory effect. Second, it 30 is generally desirable that the oligonucleotide and its target be sequence specific. Third, the oligonucleotide should have a sufficient half-life under in vivo

1 conditions for it to be able to accomplish its desired regulatory action in the cell. Hence, the oligonucleotide should be resistant to enzymes that degrade nucleic acids, e.g. nucleases. Fourth, the 5 oligonucleotide should be able to bind to single- and double-stranded targets.

While linear oligonucleotides may satisfy the requirement for sequence specificity, linear oligonucleotides are sensitive to nucleases and 10 generally require chemical modification to increase biological half-life. Such modifications increase the cost of making an oligonucleotide and may present toxicity-problems. Furthermore, linear oligonucleotides bind to form a two-stranded complex like those present 15 in cellular nucleic acids. Consequently, cellular enzymes can readily manipulate and dissociate a linear oligonucleotide bound in a double-stranded complex with target. The low binding strength and nuclease sensitivity of linear oligonucleotides can thus 20 necessitate administration of high concentrations of oligonucleotide, in turn making such administration toxic or costly. Moreover, while linear oligonucleotides can bind to a double-stranded target via alternate hydrogen bonds (e.g. Hoogsteen binding), 25 linear oligonucleotides cannot readily dissociate a double-stranded target to replace one strand and thereby form a more stable Watson-Crick bonding pattern.

Furthermore, increased binding strength increases the effectiveness of a regulatory 30 oligonucleotide. Therefore, an oligonucleotide with high binding affinity can be used at lower dosages. Lower dosages decrease costs and reduce the likelihood

1 Accordingly, the present invention provides single-stranded circular oligonucleotides which, by nature of the circularity of the oligonucleotide and the domains present on the oligonucleotide, are nuclease 5 resistant and bind with strong affinity and high selectivity to their targeted nucleic acids. Moreover, the present circular oligonucleotides can dissociate and bind to a double-stranded target without prior denaturation of that target.

10 Some types of single-stranded circles of DNA or RNA are known. For example, the structures of some naturally occurring viral and bacteriophage genomes are single-stranded circular nucleic acids. Single-stranded circles of DNA have been studied by Erie et al. (1987, 15 *Biochemistry* 26: 7150-7159 and 1989, *Biochemistry* 28: 268-273). However, none of these circular molecules are designed to bind a target nucleic acid. Hence, the present invention represents an innovation characterized by a substantial improvement relative to the prior art 20 since the subject circular oligonucleotides exhibit high specificity, low or no toxicity and more resistance to nucleases than linear oligonucleotides, while binding to single- or double-stranded target nucleic acids more strongly than conventional linear oligonucleotides.

25

SUMMARY OF THE INVENTION:

The present invention provides a single-stranded circular oligonucleotide having at least one parallel binding (P) domain and at least one anti-parallel binding (AP) domain, and having a loop domain 30 between each binding domain to form the circular oligonucleotide. Each P and corresponding AP domain has

1 sufficient complementarity to bind detectably to one strand of a defined nucleic acid target with the P domain binding in a parallel manner to the target, and the AP domain binding in an anti-parallel manner to the 5 target. Sufficient complementarity means that a sufficient number of base pairs exists between the target nucleic acid and the P and/or AP domains of the circular oligonucleotide to achieve stable, i.e. detectable, binding.

10 Another aspect of the present invention provides the subject single-stranded circular oligonucleotides derivatized with a reporter molecule to provide a probe for a target nucleic acid, or with a drug or other pharmaceutical agent to provide cell 15 specific drug delivery, or with agents which can cleave or otherwise modify the target nucleic acid or, furthermore, with agents that can facilitate cellular uptake or target binding of the oligonucleotide.

An additional aspect of the present invention 20 provides single-stranded circular oligonucleotides linked to a solid support for isolation of a nucleic acid complementary to the oligonucleotide.

Another aspect of the present invention provides a compartmentalized kit for detection or 25 diagnosis of a target nucleic acid including at least one first container providing any one of the present circular oligonucleotides.

A further aspect of the present invention provides a method of detecting a target nucleic acid 30 which involves contacting a single-stranded circular oligonucleotide with a sample containing the target nucleic acid, for a time and under conditions sufficient

1 to form an oligonucleotide-target complex, and detecting the complex. This detection method can be by fluorescent energy transfer.

5 A still further aspect of the present invention provides a method of regulating biosynthesis of a DNA, an RNA or a protein. This method includes contacting at least one of the subject circular oligonucleotides with a nucleic acid template for the DNA, the RNA or the protein under conditions sufficient 10 to permit binding of the oligonucleotide to a target sequence contained in the template, followed by binding of the oligonucleotide to the target, blocking access to the template and thereby regulating biosynthesis of the DNA, the RNA or the protein.

15 An additional aspect of the present invention provides pharmaceutical compositions for regulating biosynthesis of a nucleic acid or protein containing a biosynthesis regulating amount of at least one of the subject circular oligonucleotides and a pharmaceutically acceptable carrier.

20 A further aspect of the present invention provides a method of preparing a single-stranded circular oligonucleotide which includes binding a linear precircle to an end-joining-oligonucleotide, joining the 25 ~~two ends of the precircle and recovering the circular~~ oligonucleotide product.

25 Another aspect of the present invention provides a method of strand displacement in a double-stranded nucleic acid target by contacting the target 30 with any one of the present circular oligonucleotides for a time and under conditions effective to denature the target and to bind the circular oligonucleotide.

1 BRIEF DESCRIPTIONS OF THE DRAWINGS:

Fig. 1A depicts the bonding patterns of Watson-Crick (anti-parallel domain) AT and GC base pairs. Fig. 1B depicts T-AT, C+GC and G-TA base triads 5 that can form between P, target and AP nucleotides.

Fig. 2 schematically illustrates a circularization reaction for synthesis of single-stranded circular oligonucleotides. A linear precircle oligonucleotide is bound to an oligonucleotide having 10 the same sequence as the target, i.e. an end-joining-oligonucleotide, to form a precircle complex. After ligation, the circularized oligonucleotides are separated from the end-joining-oligonucleotide.

Fig. 3 depicts the sequence of linear 15 precursors to circular oligonucleotides, i.e. precircles (1-3 having SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7), targets (4,5 having SEQ ID NO: 8 and SEQ ID NO: 9), circular oligonucleotides (6,7,8 and 13 having SEQ ID NO: 5-7 and 14), and linear oligonucleotides (9-12 and 20 14 having SEQ ID NO: 10-13 and 15) described in the Examples.

Fig. 4 depicts the structure of a linear precircle complexed with an end-joining-oligonucleotide before ligation.

25 Fig. 5 depicts the effect of pH on circular oligonucleotide:target complex formation as measured by Tm. Filled circles represent the stability at different pH values for a 6:4 complex while filled squares depict the stability of a 7:5 complex. The sequences of 30 circular oligonucleotides 6 and 7 and targets 4 and 5 are presented in Fig. 3.

1 Fig. 6A depicts the effect of loop size on
complex formation, with a comparison between binding to
two targets: a simple $(dA)_{12}$ target (squares) and a 36
5 nucleotide oligonucleotide target (circles). Fig. 6B
5 depicts the effect of target and binding domain length
on complex formation.

Fig. 7 depicts a complex formed between a
circular oligonucleotide and a target where the P and AP
binding domains are staggered on the target.

10 Fig. 8 depicts replacement of one strand of a
fluorecently labeled double stranded target (SEQ ID NO:
11) by either a linear oligonucleotide having SEQ ID NO:
8 (dotted line) or a circular oligonucleotide having SEQ
ID NO: 5 (solid line). Strand replacement was measured
15 by an increase in fluorescein fluorescence intensity (Y-
axis) as a function of time (X-axis).

Fig. 9 depicts a plot of observed pseudo-first
order rate constant, K_{obs} for duplex target (SEQ ID NO:
5) at several concentrations. Uncertainty in rate
20 constants are no more than $\pm 10\%$. The depicted curve is
a rectangular hyperbola generated as a best fit. A
double reciprocal plot of the data, i.e., $[\text{circular}$
 $25 \text{ oligonucleotide}]^{-1}$ vs $(K_{obs})^{-1}$ is linear with a slope of
 $8.95 \times 10^{-6} \text{ sec} \cdot M^{-1}$ and a y-intercept of 39.8 sec.

25

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to single-
stranded circular oligonucleotides, i.e. circles, which
can bind to nucleic acid targets with higher affinity
30 and selectivity than a corresponding linear
oligonucleotide. Moreover, since the present circles
can open up two strands of a double-stranded nucleic

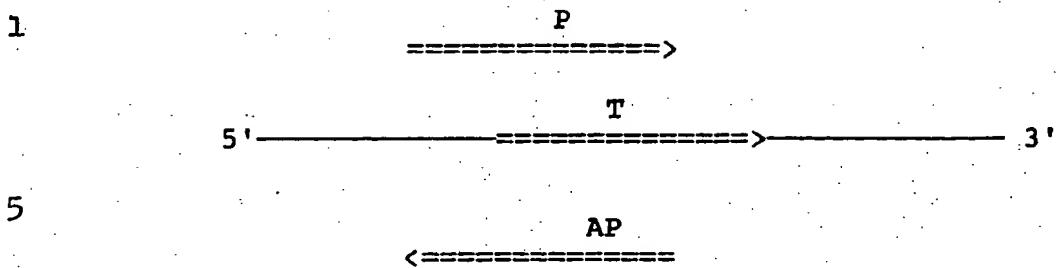
1 acid and bind thereto, both single- and double-stranded nucleic acids can be targets for binding by the present circular oligonucleotides.

Furthermore, the strong, selective binding of 5 these circles to either single- or double-stranded targets provides a variety of uses, including methods of regulating such biological processes as DNA replication, RNA transcription, RNA splicing and processing, protein translation and the like. Similarly, the ability of 10 these circles to dissociate double-stranded nucleic acids and to selectively and stably bind to targeted nucleic acids makes them ideal as diagnostic probes or as markers to localize, for example, specific sites in a chromosome or other DNA or RNA molecules. Additionally, 15 the present circles are useful for isolation of complementary nucleic acids or for sequence-specific delivery of drugs or other molecules into cells.

In particular, the single-stranded circular oligonucleotides of the present invention have at least 20 one parallel binding (P) domain and at least one anti-parallel binding (AP) domain and have a loop domain between each binding domain, so that a circular oligonucleotide is formed. Moreover, each P and AP domain exhibits sufficient complementarity to bind to 25 one strand of a defined nucleic acid target with the P domain binding to the target in a parallel manner and the AP domain binding to the target in an anti-parallel manner.

The schematic illustration set forth below 30 shows the circular arrangement of one set of P and AP oligonucleotide domains relative to each other as well as when bound to a target (T, as indicated below).

-12-



The arrows indicate the 5' to 3' orientation of each strand with the 5' end of each domain at the tail and the 3' end at the arrowhead. Hence as used herein

10 binding of nucleic acids in a parallel manner means that the 5'-to-3'-orientation is the same for each strand or nucleotide in the complex. This is the type of binding present between the target and the P domain. As used herein, binding of nucleic acids in an anti-parallel

15 manner means that the 5' to 3' orientations of two strands or nucleotides in a complex lie in opposite directions, i.e. the strands are aligned as found in the typical Watson-Crick base pairing arrangement of double helical DNA.

20 When more than one P or AP binding domain is present, such binding domains are separated from other P and AP domains by loop domains whose lengths are sufficient to permit binding to multiple targets.

Moreover, when a circle has multiple AP and P domains,

25 the corresponding targets need not be linked on one nucleic acid strand. Furthermore, a loop domain of a circular oligonucleotide bound to a given target can be an AP or P domain for binding to a second target when the circular oligonucleotide releases from the first

30 target.

1 In accordance with this invention, the
nucleotide sequences of the P and AP domains can be
det rmined from the defined sequence of the nucleic acid
target by reference to the base pairing rules provided
5 hereinbelow. A target can be either single- or double-
stranded and is selected by its known functional and
structural characteristics. For example, some preferred
targets can be coding regions, origins of replication,
reverse transcriptase binding sites, transcription
10 regulatory elements, RNA splicing junctions, or ribosome
binding sites, among others. A target can also be
selected by its capability for detection or isolation of
a DNA or RNA template. Preferred targets are rich in
purines, i.e. in adenines and guanines.

15 The nucleotide sequence of the target DNA or
RNA can be known in full or in part. When the target
nucleotide sequence is completely known the sequences of
the P and AP domains are designed with the necessary
degree of complementarity to achieve binding, as
20 detected by known procedures, for example by a change in
light absorption or fluorescence. In some instances,
the target sequence can be represented by a consensus
sequence or be only partially known. For example,
circular oligonucleotides (circles) which bind to an
25 entire class of targets represented by a consensus
sequence can be provided by designing the P and AP
domains from the target consensus sequence. In this
instance some of the targets may match the consensus
sequence exactly and others may have a few mismatched
bases, but not enough mismatch to prevent binding.
30 Likewise, if a portion of a target sequence is known,
one skilled in the art can refer to the base pairing

-14-

1 rules provided hereinbelow to design circles which bind to that target with higher affinity than a linear oligonucleotide that has a sequence corresponding to that of the circle.

5 Thus, the present invention is also directed to circles having P and AP domains which are sufficiently complementary to bind to a nucleic acid target wherein a sufficient number, but not necessarily all, nucleotide positions in the P and AP domains are 10 determined from the target sequence in accordance with the base pairing rules of this invention. The number of determined (i.e. known) positions is that number of positions which are necessary to provide sufficient complementarity for binding of the subject 15 oligonucleotides to their targets, as detected by standard procedures including a change in light absorption upon binding or melting.

The base pairing rules of the present invention provide for the P domain to bind to the target 20 by forming base pairs wherein the P domain and target nucleotides have the same 5' to 3' orientation. In particular, these rules are satisfied to the extent needed to achieve binding of a circular oligonucleotide to its nucleic acid target, i.e. the degree of 25 complementarity need not be 100% so long as binding can be detected. Hence, the general rules for determining the sequence of the P domain are thus:

when a base for a position in the target is guanine or a guanine analog, then P has cytosine, or a 30 suitable analog thereof, in a corresponding position;

when a base for a position in the target is adenine, or an adenine analog then P has thymine or

1 uracil, or suitable analogs thereof, in a corresponding position;

when a base for a position in the target is thymine, or a thymine analog, then P has cytosine or 5 guanine, or suitable analogs thereof, in a corresponding position;

when a base for a position in the target is cytosine, or a cytosine analog, then P has cytosine, thymine or uracil, or suitable analogs thereof, in a 10 corresponding position; and

when a base for a position in the target is uracil, or a uracil analog, then P has cytosine, guanine, thymine, or uracil, or suitable analogs thereof, in a corresponding position.

15 The base pairing rules of the present invention provide for the AP domain to bind to the target by forming base pairs wherein the AP domain and target nucleotides are oriented in opposite directions. In particular these rules are satisfied to the extent 20 necessary to achieve detectable binding of a circular oligonucleotide to its nucleic acid target, i.e. the degree of complementarity can be less than 100%. Hence, the base pairing rules can be adhered to only insofar as 25 is necessary to achieve sufficient complementarity for binding to be detected between the circular oligonucleotide and its target.

Thus, the general rules for determining the sequence of the AP domain are as follows:

when a base for a position in the target is 30 guanine, or a guanine analog, then AP has cytosine or uracil, or suitable analogs thereof, in a corresponding position;

1 when a base for a position in the target is adenine, or an adenine analog, then AP has thymine or uracil, or suitable analogs thereof, in a corresponding position;

5 when a base for a position in the target is thymine, or a thymine analog, then AP has adenine, or a suitable analog thereof, in a corresponding position; and

10 when a base for a position in the target is cytosine, or a cytosine analog, then AP has a guanine, or a suitable analog thereof, in a corresponding position;

15 when a base for a position in the target is uracil, or a uracil analog, then AP has adenine or guanine, or suitable analogs thereof, in a corresponding position.

In a preferred embodiment, the P, AP and loop domains are not complementary to each other.

Table 1 summarizes which nucleotides can form anti-parallel base pairs or parallel base pairs with a 20 defined target nucleotide.

Table 1

Target Nucleotide ^a	Anti-Parallel Domain Nucleotide ^a	Parallel Domain Nucleotide ^a
G	C or U	C
A	T or U	T or U
T	A	C or G
C	G	C, T or U
U	A or G	C, G, T or U

30 ^a Or a suitable analog

1 Two complementary single-stranded nucleic acids form a stable double helix (duplex) when the strands bind, or hybridize, to each other in the typical Watson-Crick fashion, i.e. via anti-parallel GC and AT 5 base pairs. For the present invention, stable duplex formation and stable triplex formation is achieved when the P and AP domains exhibit sufficient complementarity to the target sequence to achieve stable binding between the circular oligonucleotide and the target molecule. 10 Stable binding occurs when an oligonucleotide remains detectably bound to target under the required conditions.

Complementarity between nucleic acids is the degree to which the bases in one nucleic acid strand can 15 hydrogen bond, or base pair, with the bases in a second nucleic acid strand. Hence, complementarity can sometimes be conveniently described by the percentage, i.e. proportion, of nucleotides which form base pairs between two strands or within a specific region or 20 domain of two strands. For the present invention sufficient complementarity means that a sufficient number of base pairs exist between a target nucleic acid and the P and/or AP domains of the circular oligonucleotide to achieve detectable binding.

25 Moreover, the degree of complementarity between the P domain and the target and the AP domain and the target need not be the same. When expressed or measured by percentage of base pairs formed, the degree of complementarity can range from as little as about 30-40% 30 complementarity to full, i.e. 100%, complementarity. In general, the overall degree of complementarity between the P or AP domain and the target is preferably at least

1 about 50%. However, the P domain can sometimes have
less complementarity with the target than the AP domain
has with the target, for example the P domain can have
about 30% complementarity with the target while the AP
5 domain can have substantially more complementarity, e.g.
50% to 100% complementarity.

Moreover, the degree of complementarity that
provides detectable binding between the subject circular
oligonucleotides and their respective targets, is
10 dependent upon the conditions under which that binding
occurs. It is well known that binding, i.e.
hybridization, between nucleic acid strands depends on
factors besides the degree of mismatch between two
sequences. Such factors include the GC content of the
15 region, temperature, ionic strength, the presence of
formamide and types of counter ions present. The effect
that these conditions have upon binding is known to one
skilled in the art. Furthermore, conditions are
frequently determined by the circumstances of use. For
20 example, when a circular oligonucleotide is made for use
in vivo, no formamide will be present and the ionic
strength, types of counter ions, and temperature
correspond to physiological conditions. Binding
conditions can be manipulated in vitro to optimize the
25 utility of the present oligonucleotides. A thorough
treatment of the qualitative and quantitative
considerations involved in establishing binding
conditions that allow one skilled in the art to design
appropriate oligonucleotides for use under the desired
30 conditions is provided by Beltz et al., 1983, Methods
Enzymol. 100: 266-285 and by Sambrook et al.

1 Thus for the present invention, one of
ordinary skill in the art can readily design a
nucleotide sequence for the P and AP domains of the
subject circular oligonucleotides which exhibits
5 sufficient complementarity to detectably bind to its
target sequence. As used herein "binding" or "stable
binding" means that a sufficient amount of the
oligonucleotide is bound or hybridized to its target to
permit detection of that binding. Binding can be
10 detected by either physical or functional properties of
the target:circular oligonucleotide complex.

Binding between a target and an
oligonucleotide can be detected by any procedure known
to one skilled in the art, including both functional or
15 physical binding assays. Binding may be detected
functionally by determining whether binding has an
observable effect upon a biosynthetic process such as
DNA replication, RNA transcription, protein translation
and the like.

20 Physical methods of detecting the binding of
complementary strands of DNA or RNA are well known in
the art, and include such methods as DNase I or chemical
footprinting, gel shift and affinity cleavage assays and
light absorption detection procedures. For example, a
25 method which is widely used, because it is so simple and
reliable, involves observing a change in light
absorption of a solution containing an oligonucleotide
and a target nucleic acid at 220 to 300 nm as the
temperature is slowly increased. If the oligonucleotide
30 has bound to its target, there is a sudden increase in
absorption at a characteristic temperature as the
oligonucleotide and target dissociate or melt.

1 The binding between an oligonucleotide and its target nucleic acid is frequently characterized by the temperature at which 50% of the oligonucleotide is melted from its target. This temperature is the melting 5 temperature (T_m). A higher T_m means a stronger or more stable complex relative to a complex with a lower T_m . The stability of a duplex increases with increasing G:C content since G:C base pairs have three hydrogen bonds whereas A:T base pairs have two. The circular 10 oligonucleotides of the present invention provide additional hydrogen bonds and hence more stability since two binding domains are available for bonding to a single target nucleic acid, i.e. the P domain and the AP domain. Hence, the triplex formed by a circular 15 oligonucleotide bound to its target nucleic acid should melt at a higher T_m than the duplex formed by a linear oligonucleotide and a target.

20 Circular oligonucleotides bind to a nucleic acid target through hydrogen bonds formed between the nucleotides of the binding domains and the target. The AP domain can bind by forming Watson-Crick hydrogen bonds (Fig. 1). The P domain can bind to the target nucleotides by forming non-Watson-Crick hydrogen bonds (e.g., Fig. 1 and Table I). When two nucleotides from 25 different strands of DNA or RNA hydrogen bond by the base pairing rules defined herein, a base pair or duplex is formed. When a nucleotide from AP and a nucleotide from P both bind to the same target nucleotide, a base triad is formed.

30 Parallel domain base pairing with a complementary target strand of nucleic acid, is thermodynamically less favorable than Watson-Crick base

1 pairing; however, when both parallel and antiparallel pairing modes are present in a single molecule, highly stable complexs can form. Thus, two opposing domains of a circular oligomer form a complex with a central 5 target, giving a triplex structure, or a triple helical complex, bounded by the two looped ends of the circle. For example, this arrangement can allow formation of up to four hydrogen bonds when two thymines bind to a target adenine and up to five hydrogen bonds when two 10 cytosines bind to a target guanine.

Furthermore, because of the binding characteristics of the P and AP domains, the present circular oligonucleotides have a higher selectivity for a particular target than do corresponding linear 15 oligonucleotides. At least two factors can contribute to this high selectivity. First, circular oligonucleotides of this invention bind twice to the same central target strand. Hence two domains are involved in selecting a target. Second, protonation of 20 cytosine in a C+G-C triad is favored only when this triad forms and the additional proton gives the triad a positive charge. This positive charge can lessen the negative charge repulsions arising from the juxtapositioning of three phosphodiester backbones.

25 Unlike linear oligonucleotides, the present circular oligonucleotides can displace one strand of a double-stranded target under conditions where denaturation of the double-stranded target is thermodynamically unfavorable. Linear oligonucleotides 30 do not have this capacity to displace a strand of a duplex. For example, the half-life of a double-stranded target in the presence of a complementary linear

1 oligonucleotide is about 58 min i. . so long that the linear oligonucleotide has little utility for displacing one strand of the duplex target. However, a double-stranded target has a half-life of only 30 sec in the 5 presence of the present circular oligonucleotides. Therefore, the circular oligonucleotides of the present invention have utility not only for binding single-stranded targets, but also for binding to double-stranded targets. Accordingly, since both single- and 10 double-stranded nucleic acids are available as targets for the present circular oligonucleotides, these circular oligonucleotides can have greater utility than linear oligonucleotides. For example, the present circular oligonucleotides are better regulators of 15 biological processes in vivo and better in vitro diagnostic probes than corresponding linear oligonucleotides.

When the nucleic acid template extends beyond the central triple-stranded target:circle complex, a P 20 or an AP domain may bind as duplex on either side of the triple standard complex. Hence a target:circular oligonucleotide complex can be partially two stranded and partially three-stranded, wherein two-stranded portions can be P:target duplexes, without bound AP 25 nucleotides, or AP:target duplexes, without bound P nucleotides. This binding arrangement is a staggered binding arrangement.

Each P domain, AP domain and target can independently have about 2 to about 200 nucleotides with 30 preferred lengths being about 4 to about 100 nucleotides. The most preferred lengths are 6 to 36 nucleotides.

1 The P and AP domains are separated by loop domains which can independently have from about 2 to about 2000 nucleotides. A preferred loop length is from about 3 to about 8 nucleotides with an especially 5 preferred length being about 5 nucleotides.

According to the present invention, the loop domains do not have to be composed of nucleotide bases. Non-nucleotide loops can make the present circular oligonucleotides cheaper to produce. More 10 significantly, circular oligonucleotides with non-nucleotide loops are more resistant to nucleases and therefore have a longer biological half-life than linear oligonucleotides. Furthermore, loops having no charge, or a positive charge, can be used to promote 15 binding by eliminating negative charge repulsions between the loop and target. In addition, circular oligonucleotides having uncharged or hydrophobic non-nucleotide loops can penetrate cellular membranes better than circular oligonucleotides with nucleotide loops.

20 As contemplated herein, non-nucleotide loop domains can be composed of alkyl chains, polyethylene glycol or oligoethylene glycol chains or other chains providing the necessary steric or flexibility properties which are compatible with oligonucleotide synthesis.

25 The length of these chains is equivalent to about 2 to about 2000 nucleotides, with preferred lengths equivalent to about 3 to about 8 nucleotides. The most preferred length for these chains is equivalent to about 5 nucleotides.

30 Preferred chains for non-nucleotide loop domains are polyethylene glycol or oligoethylene glycol chains. In particular, oligoethylene glycol chains

1 having a length similar to a 5 nucleotide chain, e.g. a pentaethylene glycol, a hexaethylene glycol or a heptaethylene glycol chain, are preferred.

The circular oligonucleotides are single-stranded DNA or RNA, with the bases guanine (G), adenine (A), thymine (T), cytosine (C) or uracil (U) in the nucleotides, or with any nucleotide analog that is capable of hydrogen bonding in a parallel or anti-parallel manner. Nucleotide analogs include

10 pseudouridine, isopseudouridine, 3-aminophenyl-imidazole, 2'-O-methyl-adenosine, 7-deazadenosine, 7-

deazaguanosine, 4-acetylcytidine, 5-(carboxy-hydroxymethyl)-uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl-2-thioridine, 5-

15 carboxymethylamino-methyluridine, dihydrouridine, 2'-O-methyluridine, 2'-O-methyl-pseudouridine, beta,D-galactosylqueosine, 2'-O-methylguanosine, inosine, N6-isopentenyladenosine, 1-methyladenosine, 1-methyl-pseudouridine, 1-methylguanosine, 1-methylinosine, 2,2-

20 dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, 5-methyluridine, N6-methyl-adenosine, 7-methylguanosine, 5-methylamino-methyluridine, 5-methoxyaminomethyl-2-thiouridine, 8-D-mannosylqueosine, 5-methoxycarbonylmethyluridine, 5-

25 methoxyuridine, 2-methyl-thio-N6-isopentenyladenosine, N-(9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)-carbamoyl)threonine, N-(9-beta-D-ribofuranosylpurine-6-yl)-N-methylcarbamoyl)threonine. When possible, either ribose or deoxyribose sugars can be used with these

30 analogs. Nucleotides bases in an α -anomeric conformation can also be used in the circular oligonucleotides of the present invention.

1 Preferred nucleotide analogs are unmodified G, A, T, C and U nucleotides; pyrimidine analogs with lower alkyl, lower alkoxy, lower alkylamine, phenyl or lower alkyl substituted phenyl groups in the 5 position of the 5 base and purine analogs with similar groups in the 7 or 8 position of the base. Especially preferred nucleotide analogs are 5-methylcytosine, 5-methyluracil, diaminopurine, and nucleotides with a 2'-0-methylribose moiety in place of ribose or deoxyribose. As used 10 herein lower alkyl, lower alkoxy and lower alkylamine contain from 1 to 6 carbon atoms and can be straight chain or branched. These groups include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tertiary butyl, amyl, hexyl and the like. A preferred alkyl group is 15 methyl.

15 Circular oligonucleotides can be made first as linear oligonucleotides and then circularized. Linear oligonucleotides can be made by any of a myriad of procedures known for making DNA or RNA oligonucleotides. 20 For example, such procedures include enzymatic synthesis and chemical synthesis.

25 Enzymatic methods of DNA oligonucleotide synthesis frequently employ Klenow, T7, T4, Tag or E. coli DNA polymerases as described in Sambrook et al.

Enzymatic methods of RNA oligonucleotide synthesis frequently employ SP6, T3 or T7 RNA polymerase as described in Sambrook et al. Reverse transcriptase can also be used to synthesize DNA from RNA (Sambrook et al.). To prepare oligonucleotides enzymatically 30 requires a template nucleic acid which can either be synthesized chemically, or be obtained as mRNA, genomic DNA, cloned genomic DNA, cloned cDNA or other

1 recombinant DNA. Some enzymatic methods of DNA oligonucleotide synthesis can require an additional primer oligonucleotide which can be synthesized chemically. Finally, linear oligonucleotides can be 5 prepared by PCR techniques as described, for example, by Saiki et al., 1988, Science 239:487.

Chemical synthesis of linear oligonucleotides is well known in the art and can be achieved by solution or solid phase techniques. Moreover, linear 10 oligonucleotides of defined sequence can be purchased commercially or can be made by any of several different synthetic procedures including the phosphoramidite, phosphite triester, H-phosphonate and phosphotriester methods, typically by automated synthesis methods. The 15 synthesis method selected can depend on the length of the desired oligonucleotide and such choice is within the skill of the ordinary artisan. For example, the phosphoramidite and phosphite triester method produce oligonucleotides having 175 or more nucleotides while 20 the H-phosphonate method works well for oligonucleotides of less than 100 nucleotides. If modified bases are incorporated into the oligonucleotide, and particularly if modified phosphodiester linkages are used, then the synthetic procedures are altered as needed according to 25 known procedures. In this regard, Uhlmann et al. (1990, Chemical Reviews 90: 543-584) provide references and outline procedures for making oligonucleotides with modified bases and modified phosphodiester linkages.

Synthetic, linear oligonucleotides may be 30 purified by polyacrylamide gel electrophoresis, or by any of a number of chromatographic methods, including gel chromatography and high pressure liquid

1 chromatography. To confirm a nucleotide sequence, oligonucleotides may be subjected to DNA sequencing by any of the known procedures, including Maxam and Gilbert sequencing, Sanger sequencing, capillary electrophoresis 5 sequencing the wandering spot sequencing procedure or by using selective chemical degradation of oligonucleotides bound to Hybond paper. Sequences of short oligonucleotides can also be analyzed by plasma desorption mass spectroscopy or by fast atom bombardment 10 (McNeal, et al., 1982, J. Am. Chem. Soc. 104: 976; Viari, et al., 1987, Biomed. Environ. Mass Spectrom. 14: 83; Grotjahn et al., 1982, Nuc. Acid Res. 10: 4671). Sequencing methods are also available for RNA oligonucleotides.

15 The present invention provides several methods of preparing circular oligonucleotides from linear precursors (i.e. precircles), including a method wherein a precircle is synthesized and bound to an end-joining-oligonucleotide and the two ends of the precircle are 20 joined. Any method of joining two ends of an oligonucleotide is contemplated by the present invention, including chemical methods employing, for example, known coupling agents like BrCN, N-cyanoimidazole ZnCl₂, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and other carbodiimides 25 and carbonyl diimidazoles. Furthermore, the ends of a precircle can be joined by condensing a 5' phosphate and a 3' hydroxy, or a 5' hydroxy and a 3' phosphate.

In accordance with the present invention, a 30 simple one-step chemical method is provided to construct the subject circular oligonucleotides, or circles, from precircles. An oligonucleotide is constructed which has

1 the same sequence as the target nucleic acid; this is the end-joining oligonucleotide. A DNA or RNA linear precircle is chemically or enzymatically synthesized and phosphorylated on its 5' or 3' end, again by either 5 chemical or enzymatic means. The precircle and the end-joining oligonucleotide are mixed and annealed, thereby forming a complex in which the 5' and 3' ends of the precircle are adjacent, as depicted in Fig. 2. It is preferred that the ends of the precircle fall within a 10 binding domain, not within a loop, and preferably within the anti-parallel binding domain rather than the parallel domain. Moreover, it is preferred that a precircle have a 3'-phosphate rather than a 5'-phosphate. After complex formation, the ends undergo a 15 condensation reaction in a buffered aqueous solution containing divalent metal ions and BrCN at about pH 7.0. In a preferred embodiment the buffer is imidazole-Cl at pH 7.0 with a divalent metal such as Ni, Zn, Mn, or Co. Ni is the most preferred divalent metal. Condensation 20 occurs after about 6-48 hr. of incubation at 4-37°C. Other divalent metals, such as Cu, Pb, Ca and Mg, can also be used.

One method for RNA circularization incorporates the appropriate nucleotide sequences, 25 preferably in a loop domain, into an RNA oligonucleotide to promote self splicing, since a circular product is formed under the appropriate conditions (Sugimoto et al., 1988, *Biochemistry*: 27: 6384-6392).

Enzymatic circle closure is also possible 30 using DNA ligase or RNA ligase under conditions appropriate for these enzymes.

1 Circular oligonucleotides can be separated
from the template by denaturing gel electrophoresis or
melting followed by gel electrophoresis, size selective
chromatography, or other appropriate chromatographic or
5 electrophoretic methods. The recovered circular
oligonucleotide can be further purified by standard
techniques as needed for its use in the methods of the
present invention.

The present invention also contemplates
10 derivatization or chemical modification of the subject
oligonucleotides with chemical groups to facilitate
cellular uptake. For example, covalent linkage of a
cholesterol moiety to an oligonucleotide can improve
cellular uptake by 5- to 10- fold which in turn improves
15 DNA binding by about 10- fold (Boutorin et al., 1989,
FEBS Letters 254: 129-132). Other ligands for cellular
receptors may also have utility for improving cellular
uptake, including, e.g. insulin, transferrin and others.
Similarly, derivatization of oligonucleotides with poly-
20 L-lysine can aid oligonucleotide uptake by cells
(Schell, 1974, Biochem. Biophys. Acta 340: 323, and
Lemaitre et al., 1987, Proc. Natl. Acad. Sci. USA 84:
648). Certain protein carriers can also facilitate
cellular uptake of oligonucleotides, including, for
25 example, serum albumin, nuclear proteins possessing
signals for transport to the nucleus, and viral or
bacterial proteins capable of cell membrane penetration.
Therefore, protein carriers are useful when associated
with or linked to the circular oligonucleotides of this
30 invention. Accordingly, the present invention
contemplates derivatization of the subject circular
oligonucleotides with groups capable of facilitating

1 cellular uptake, including hydrocarbons and non-polar groups, cholesterol, poly-L-lysine and proteins, as well as other aryl or steroid groups and polycations having analogous beneficial effects, such as phenyl or naphthyl 5 groups, quinoline, anthracene or phenanthracene groups, fatty acids, fatty alcohols and sesquiterpenes, diterpenes and steroids.

The present invention further contemplates derivatization of the subject oligonucleotides with 10 agents that can cleave or modify the target nucleic acid or other nucleic acid strands associated with or in the vicinity of the target. For example, viral DNA or RNA can be targeted for destruction without harming cellular nucleic acids by administering a circular 15 oligonucleotide complementary to the targeted nucleic acid which is linked to an agent that, upon binding, can cut or render the viral DNA or RNA inactive. Nucleic acid destroying agents that are contemplated by the present invention as having cleavage or modifying 20 activities include, for example, RNA and DNA nucleases, ribozymes that can cleave RNA, azidoproflavine, acridine, EDTA/Fe, chloroethylamine, azidophenacyl and phenanthroline/Cu. Uhlmann et al. (1990, Chemical Reviews 90: 543-584) provide further information on the 25 use of such agents and methods of derivatizing oligonucleotides that can be adapted for use with the subject circular oligonucleotides.

Derivatization of the subject circular oligonucleotides with groups that facilitate cellular 30 uptake or target binding, as well as derivatization with nucleic acid destroying agents or drugs, can be done by any of the procedures known to one skilled in the art.

1 Moreover, the desired groups can be added to nucleotid s
before synthesis of the oligonucleotide. For example,
these groups can be linked to the 5-position of T or C
and these modified T and C nucleotides can be used for
5 synthesis of the present circular oligonucleotides. In
addition, derivatization of selected nucleotides permits
incorporation of the group into selected domains of the
circular oligonucleotide. For example, in some
instances it is preferable to incorporate certain groups
10 into a loop where that group will not interfere with
binding, or into an AP or P domain to facilitate
cleavage or modification of the target nucleic acid.

In accordance with the present invention,
modification in the phosphodiester backbone of circular
15 oligonucleotides is also contemplated. Such
modifications can aid uptake of the oligonucleotide by
cells or can extend the biological half-life of such
nucleotides. For example, circular oligonucleotides may
penetrate the cell membrane more readily if the negative
20 charge on the internucleotide phosphate is eliminated.
This can be done by replacing the negatively charged
phosphate oxygen with a methyl group, an amine or by
changing the phosphodiester linkage into a
phosphotriester linkage by addition of an alkyl group to
25 the negatively charged phosphate oxygen. Alternatively,
one or more of the phosphate atoms which is part of the
normal phosphodiester linkage can be replaced. For
example, NH-P, CH₂-P or S-P linkages can be formed.
Accordingly, the present invention contemplates using
30 methylphosphonates, phosphorothioates,
phosphorodithioates, phosphotriesters and phosphorus-
boron (Sood et al., 1990, J. Am. Chem. Soc. 112: 9000)

1 linkages. The phosphodiester group can be replaced with siloxane, carbonate, acetamide or thioether groups. These modifications can also increase the resistance of the subject oligonucleotides to nucleases. Methods for 5 synthesis of oligonucleotides with modified phosphodiester linkages are reviewed by Uhlmann *et al.*

Circular oligonucleotides with non-nucleotide loops can be prepared by any known procedure. For example, Durand *et al.* (1990, *Nucleic Acids Res.* **18**: 10 6353-6359) provides synthetic procedures for linking non-nucleotide chains to DNA. Such procedures can generally be adapted to permit an automated synthesis of a linear oligonucleotide precursor which is then used to make a circular oligonucleotide of the present 15 invention. In general, groups reactive with nucleotides in standard DNA synthesis, e.g. phosphoramidite, H-phosphonate, dimethoxytrityl, monomethoxytrityl and the like, can be placed at the ends of non-nucleotide chains and nucleotides corresponding to the ends of P and AP domains can be linked thereto.

20 Additionally, different nucleotide sugars can be incorporated into the oligonucleotides of this invention. For example, RNA oligonucleotides can be used since RNA:DNA hybrids are more stable than DNA:DNA 25 hybrids. Additional binding stability can also be provided by using 2'-O-methyl ribose in the present circular oligonucleotides. Phosphoramidite chemistry can be used to synthesize RNA oligonucleotides as described (Reese, C. B. In Nucleic Acids & Molecular 30 Biology; Springer-Verlag: Berlin, 1989; Vol. 3, p. 164; and Rao, *et al.*, 1987, *Tetrahedron Lett.* **28**: 4897).

1 The synthesis of RNA 2'-0-methyl-
oligoribonucleo-tides and DNA oligonucleotides differ
only slightly. RNA 2'-0-methyloligonucleotides can be
prepared with minor modifications of the amidite, H-
5 phosphonate or phosphotriester methods (Shibahara et al.,
1987, Nucleic Acids Res. 15: 4403; Shibahara et al.,
1989, Nucleic Acids Res. 17: 239; Anoue et al., 1987,
Nucleic Acids Res. 15: 6131).

10 In another embodiment the present invention,
circular oligonucleotides can accelerate the
dissociation of a double-stranded nucleic acid target.
Therefore the double-stranded nucleic acid target does
not have to be subjected to denaturing conditions before
binding of the present circular oligonucleotides. Thus,
15 the circular oligonucleotides can bind to both single-
and double-stranded nucleic acid targets under a wider
variety of conditions, and particularly under
physiological conditions. The present circular
oligonucleotides are several orders of magnitude faster
20 at accelerating duplex nucleic acid strand displacement
than are the corresponding linear oligonucleotides.

25 The present invention therefore provides a
means to displace one strand of a double-stranded
nucleic acid target with one of the subject circular
oligonucleotides without the necessity of prior
denaturation of the double-stranded nucleic acid target.
Thus, the present invention provides a method of strand
displacement in a double-stranded nucleic acid target by
contacting the target with one of the subject circular
30 oligonucleotides for a time and under conditions
effective to denature said target and permit the
circular oligonucleotide to bind to the target. The

1 target for the present circular oligonucleotides can be a double-stranded nucleic acid, either RNA or DNA, which has not undergone denaturation by, for example, heating or exposure to alkaline pH.

5 As used herein, the nucleic acids for strand displacement can be present in an organism or present in a sample which includes an impure or pure nucleic acid preparation, a tissue section, a prokaryotic or eukaryotic cell smear, a chromosomal squash and the 10 like. Moreover, the nucleic acid targets for strand displacement by the present circular oligonucleotides include viral, bacterial, fungal or mammalian nucleic acids.

According to the present invention, conditions 15 effective to denature the target by strand displacement and thereby permit binding, include having a suitable circular oligonucleotide to target nucleic acid ratio. Moreover, as used herein a suitable ratio of circular oligonucleotide to target is about 1 to about 100, and 20 is preferably about 1 to about 50.

Moreover, as used herein a time effective to denature a double-stranded nucleic acid by strand-displacement with an oligonucleotide of the present invention is about 1 minute to about 16 hours.

25 A circular oligonucleotide can associate with a duplex target by first binding in the P domain. Such P domain binding juxtaposes the AP domain nucleotides to compete for Watson-Crick binding to target nucleotide. This P domain pre-association followed by AP domain 30 nucleotide competition for Watson-Crick binding may form the basis for the observed acceleration in strand displacement by circular oligonucleotides.

1 In summary, the subject circular
oligonucleotides have three important features which
enable duplex strand displacement. First, the circular
oligonucleotide has the ability to preassociate, which
5 results in a high local concentration. Second, the
circular oligonucleotide contains a second (AP) binding
domain, which competes for binding to a complementary
strand of the duplex. Finally, the circular
oligonucleotide binds with higher affinity than the
10 displaced strand of the duplex, thereby driving the
reaction to completion.

The present invention contemplates a variety
of utilities for the subject circular oligonucleotides
which are made possible by their selective and stable
15 binding properties with both single- and double-stranded
targets. Some utilities include, but are not limited
to: use of circular oligonucleotides of defined
sequence, bound to a solid support, for affinity
isolation of complementary nucleic acids; use of the
20 subject oligonucleotides to provide sequence specific
stop signals during polymerase chain reaction (PCR);
covalent attachment of a drug, drug analog or other
therapeutic agent to circular oligonucleotides to allow
cell type specific drug delivery; labeling circular
25 oligonucleotides with a detectable reporter molecule for
localizing, quantitating or identifying complementary
target nucleic acids; and binding circular
oligonucleotides to a cellular or viral nucleic acid
template and regulating biosynthesis directed by that
30 template.

The subject circular oligonucleotides can be
attached to a solid support such as silica, cellulose,

1 nylon, and other natural or synthetic materials that are used to make beads, filters, and column chromatography resins. Attachment procedures for nucleic acids to solid supports of these types are well known; any known 5 attachment procedure is contemplated by the present invention. A circular oligonucleotide attached to a solid support can then be used to isolate a complementary nucleic acid. Isolation of the complementary nucleic acid can be done by incorporating 10 the oligonucleotide:solid support into a column for chromatographic procedures. Other isolation methods can be done without incorporation of the oligonucleotide:solid support into a column, e.g. by utilization of filtration procedures. Circular 15 oligonucleotide:solid supports can be used, for example, to isolate poly(A)⁺ mRNA from total cellular or viral RNA by making a circular oligonucleotide with P and AP domain poly(dT) or poly(U) sequences. Circular oligonucleotides are ideally suited to applications of 20 this type because they are nuclease resistant and bind target nucleic acids so strongly.

Further utilities are available for the subject oligonucleotides in the field of polymerase chain reaction (PCR) technology. PCR technology 25 provides methods of synthesizing a double standard DNA fragment encoded in a nucleic acid template between two known nucleic acid sequences which are employed as primer binding sites. In some instances it is desirable to produce a single-stranded DNA fragment before or 30 after having made some of the double stranded fragment. This can be done by, for example, binding a circular oligonucleotide of the present invention to one of the

1 primer binding sites or to a site lying between the primer binding sites.

The present invention also contemplates using the subject circular oligonucleotides for targeting 5 drugs to specific cell types. Such targeting can allow selective destruction or enhancement of particular cell types, e.g. inhibition of tumor cell growth can be attained. Different cell types express different genes, so that the concentration of a particular mRNA can be 10 greater in one cell type relative to another cell type, such an mRNA is a target mRNA for cell type specific drug delivery by circular oligonucleotides linked to drugs or drug analogs. Cells with high concentrations of target mRNA are targeted for drug delivery by 15 administering to the cell a circular oligonucleotide with a covalently linked drug that is complementary to the target mRNA.

The present invention also contemplates labeling the subject circular oligonucleotides for use 20 as probes to detect a target nucleic acid. Labelled circular oligonucleotide probes have utility in diagnostic and analytical hybridization procedures for localizing, quantitating or detecting a target nucleic acid in tissues, chromosomes or in mixtures of nucleic 25 acids. Circular oligonucleotide probes of this invention represent a substantial improvement over linear nucleic acid probes because the circular oligonucleotides can replace one strand of a double-stranded nucleic acid, and because the present oligonucleotides have two 30 binding domains which not only provide increased binding stability but also impart a greater sequence selectivity

1 (or specificity) for the target:oligonucleotide interaction.

Labeling of a circular oligonucleotide can be done by incorporating nucleotides linked to a "reporter molecule" into the subject circular oligonucleotides. A "reporter molecule", as defined herein, is a molecule or atom which, by its chemical nature, provides an identifiable signal allowing detection of the circular oligonucleotide. Detection can be either qualitative or 10 quantitative. The present invention contemplates using any commonly used reporter molecule including radionuclides, enzymes, biotins, psoralens, fluorophores, chelated heavy metals, and luciferin. The most commonly used reporter molecules are either 15 enzymes, fluorophores or radionuclides linked to the nucleotides which are used in circular oligonucleotide synthesis. Commonly used enzymes include horseradish peroxidase, alkaline phosphatase, glucose oxidase and β -galactosidase, among others. The substrates to be used 20 with the specific enzymes are generally chosen because a detectably colored product is formed by the enzyme acting upon the substrate. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for horseradish peroxidase, 1,2- 25 phenylenediamine, 5-aminosalicylic acid or toluidine are commonly used. The probes so generated have utility in the detection of a specific DNA or RNA target in, for example, Southern analysis, Northern analysis, in situ hybridization to tissue sections or chromosomal squashes 30 and other analytical and diagnostic procedures. The methods of using such hybridization probes are well

1 known and some examples of such methodology are provided by Sambrook et al.

5 The present circular oligonucleotides can be used in conjunction with any known detection or diagnostic procedure which is based upon hybridization of a probe to a target nucleic acid. Moreover, the present circular oligonucleotides can be used in any hybridization procedure which quantitates a target nucleic acid, e.g., by competitive hybridization between 10 a target nucleic acid present in a sample and a labeled tracer target for one of the present oligonucleotides. Furthermore, the reagents needed for making a circular oligonucleotide probe and for utilizing such a probe in a hybridization procedure can be marketed in a kit.

15 15 The kit can be compartmentalized for ease of utility and can contain at least one first container providing reagents for making a precircle precursor for a circular oligonucleotide, at least one second container providing reagents for labeling the precircle 20 with a reporter molecule, at least one third container providing reagents for circularizing the precircle, and at least one fourth container providing reagents for isolating the labeled circular oligonucleotide.

25 Moreover the present invention provides a kit for isolation of a template nucleic acid. Such a kit has at least one first container providing a circular oligonucleotide which is complementary to a target contained within the template. For example, the template nucleic acid can be cellular and/or viral 30 poly(A)⁺ mRNA and the target can be the poly(A)⁺ tail. Hence circular oligonucleotides of the present invention

1 which have utility for isolation of poly(A)+ mRNA have P and AP domain sequences of poly(dT) or poly(U).

Furthermore, the present invention provides kits useful when diagnosis of a disease depends upon 5 detection of a specific, known target nucleic acid.

Such nucleic acid targets can be, for example, a viral nucleic acid, an extra or missing chromosome or gene, a mutant cellular gene or chromosome, an aberrantly expressed RNA and others. The kits can be

10 compartmentalized to contain at least one first container providing a circular oligonucleotide linked to a reporter molecule and at least one second container providing reagents for detection of the reporter molecule.

15 One aspect of the present invention provides a method of regulating biosynthesis of a DNA, an RNA or a protein by contacting at least one of the subject circular oligonucleotides with a nucleic acid template for that DNA, that RNA or that protein in an amount and 20 under conditions sufficient to permit the binding of the oligonucleotide(s) to a target sequence contained in the template. The binding between the oligonucleotide(s) and the target blocks access to the template, and thereby regulates biosynthesis of the nucleic acid or 25 the protein. Blocking access to the template prevents proteins and nucleic acids involved in the biosynthetic process from binding to the template, from moving along the template, or from recognizing signals encoded within the template. Alternatively, when the template is RNA, 30 regulation can be accomplished by allowing selective degradation of the template. For example, RNA templates bound by the subject circular oligonucleotides are

1 susceptible to degradation by RNase H and RNase H
degradation of a select d RNA template can thereby
regulate use of the template in biosynthetic processes.

As used herein, biosynthesis of a nucleic acid
5 or a protein includes cellular and viral processes such
as DNA replication, DNA reverse transcription, RNA
transcrip-tion, RNA splicing, RNA polyadenylation, RNA
transloca-tion and protein translation, and of which can
lead to production of DNA, RNA or protein, and involve a
10 nucleic acid template at some stage of the biosynthetic
process.

As used herein, regulating biosynthesis
includes inhibiting, stopping, increasing, accelerating
or delaying biosynthesis. Regulation may be direct or
15 indirect, i.e. biosynthesis of a DNA, RNA or protein may
be regulated directly by binding a circular
oligonucleotide to the template for that DNA, RNA or
protein; alternatively, biosynthesis may be regulated
indirectly by oligonucleotide binding to a second
20 template encoding a protein that plays a role in
regulating the biosynthesis of the first DNA, RNA or
protein.

The nucleic acid templates can be RNA or DNA
and can be single-stranded or double-stranded. While
25 the present circular oligonucleotides bind to only one
strand of a target present in the template, double-
stranded templates are opened during biosynthetic
processes and thereby become available for binding.
Furthermore, the P domain of the present circular
30 oligonucleotides can bind to a double-stranded target
and place AP domain nucleotides in a position to compete
for Watson-Crick binding to target nucleotides.

1 DNA replication from a DNA template is
mediated by proteins which bind to an origin of
replication where they open the DNA and initiate DNA
synthesis along the DNA template. To inhibit DNA
5 replication in accordance with the present invention,
circular oligonucleotides are selected which bind to one
or more targets in an origin of replication. Such
binding blocks template access to proteins involved in
DNA replication. Therefore initiation and procession of
10 DNA replication is inhibited. As an alternative method
of inhibiting DNA replication, expression of the
proteins which mediate DNA replication can be inhibited
at, for example, the transcriptional or translational
level.

15 DNA replication from an RNA template is
mediated by reverse transcriptase binding to a region of
RNA also bound by a nucleic acid primer. To inhibit DNA
replication from an RNA template, reverse transcriptase
or primer binding can be blocked by binding a circular
20 oligonucleotide to the primer binding site, and thereby
blocking access to that site. Moreover, inhibition of
DNA replication can occur by binding a circular
oligonucleotide to a site residing in the RNA template
since such binding can block access to that site and to
25 downstream sites, i.e. sites on the 3' side of the
target or binding site.

30 To initiate RNA transcription, RNA polymerase
recognizes and binds to specific start sequences, or
promoters, on a DNA template. Binding of RNA polymerase
opens the DNA template. There are also additional
transcriptional regulatory elements that play a role in
transcription and are located on the DNA template.

1 These transcriptional regulatory elements include
enhancer sequences, upstream activating sequences,
repressor binding sites and others. All such promoter
and transcriptional regulatory elements, singly or in
5 combination, are targets for the subject circular
oligonucleotides. Oligonucleotide binding to these
sites can block RNA polymerase and transcription factors
from gaining access to the template and thereby
regulating, e.g., increasing or decreasing, the
10 production of RNA, especially mRNA and tRNA.

Additionally, the subject oligonucleotides can be
targeted to the coding region or 3'-untranslated region
of the DNA template to cause premature termination of
transcription. One skilled in the art can readily
15 design oligonucleotides for the above target sequences
from the known sequence of these regulatory elements,
from coding region sequences, and from consensus
sequences.

RNA transcription can be increased by, for
20 example, binding a circular oligonucleotide to a
negative transcriptional regulatory element or by
inhibiting biosynthesis of a protein that can repress
transcription. Negative transcriptional regulatory
elements include repressor sites or operator sites,
25 wherein a repressor protein binds and blocks
transcription. Oligonucleotide binding to repressor or
operator sites can block access of repressor proteins to
their binding sites and thereby increase transcription.

The primary RNA transcript made in eukaryotic
30 cells, or pre-mRNA, is subject to a number of
maturation processes before being translocated into the
cytoplasm for protein translation. In the nucleus,

1 introns are removed from the pre-mRNA in splicing reactions. The 5' end of the mRNA is modified to form the 5' cap structure, thereby stabilizing the mRNA. Various bases are also altered. The polyadenylation of 5 the mRNA at the 3' end is thought to be linked with export from the nucleus. The subject circular oligonucleotides can be used to block any of these processes.

A pre-mRNA template is spliced in the nucleus 10 by ribonucleoproteins which bind to splice junctions and intron branch point sequences in the pre-mRNA.

Consensus sequences for 5' and 3' splice junctions and for the intron branch point are known. For example, inhibition of ribonucleoprotein binding to the splice 15 junctions or inhibition of covalent linkage of the 5' end of the intron to the intron branch point can block splicing. Maturation of a pre-mRNA template can, therefore, be blocked by preventing access to these sites, i.e. by binding circular oligonucleotides of this 20 invention to a 5' splice junction, an intron branch point or a 3' splice junction. Splicing of a specific pre-mRNA template can be inhibited by using circular oligonucleotides with sequences that are complementary to the specific pre-mRNA splice junction(s) or intron 25 branch point. In a further embodiment, a collection of related splicing of pre-mRNA templates can be inhibited by using a mixture of circular oligonucleotides having a variety of sequences that, taken together, are complementary to the desired group of splice junction 30 and intron branch point sequences.

Polyadenylation involves recognition and cleavage of a pre-mRNA by a specific RNA endonuclease at

1 specific polyadenylation sites, followed by addition of a poly(A) tail onto the 3' end of the pre-mRNA. Hence, any of these steps can be inhibited by binding the subject oligonucleotides to the appropriate site.

5 RNA translocation from the nucleus to the cytoplasm of eukaryotic cells appears to require a poly(A) tail. Thus, a circular oligonucleotide is designed in accordance with this invention to bind to the poly(A) tail and thereby block access to the poly 10 (A) tail and inhibit RNA translocation. For such an oligonucleotide, both the P and AP domains can consist of about 10 to about 50 thymine residues, and preferably about 20 residues. Especially preferred P and AP domain lengths for such an oligonucleotide are about 6 to about 15 12 thymine residues.

Protein biosynthesis begins with the binding of ribosomes to an mRNA template, followed by initiation and elongation of the amino acid chain via translational "reading" of the mRNA. Protein biosynthesis, or 20 translation, can thus be blocked or inhibited by blocking access to the template using the subject circular oligonucleotides to bind to targets in the template mRNA. Such targets contemplated by this invention include the ribosome binding site (Shine- 25 Delgarno sequence), the 5' mRNA cap site, the initiation codon, and sites in the protein coding sequence. There are also classes of protein which share domains of nucleotide sequence homology. Thus, inhibition of protein biosynthesis for such a class can be 30 accomplished by targeting the homologous protein domains (via the coding sequence) with the subject circular oligonucleotides.

1 R gulation of biosynthesis by any of the
aforementioned procedures has utility for many
applications. For example, genetic disorders can be
corrected by inhibiting the production of mutant or
5 over-produced proteins, or by increasing production of
an under-expressed proteins; the expression of genes
encoding factors that regulate cell proliferation can be
inhibited to control the spread of cancer; and virally
encoded functions can be inhibited to combat viral
10 infection.

Some types of genetic disorders that can be
treated by the circular oligonucleotides of the present
invention include Alzheimer's disease, some types of
arthritis, sickle cell anemia and others. Many types of
15 viral infections can be treated by utilizing the
circular oligonucleotides of the present invention,
including infections caused by influenza, rhinovirus,
HIV, herpes simplex, papilloma virus, cytomegalovirus,
Epstein-Barr virus, adenovirus, vesticular stomatitis
20 virus, rotavirus and respiratory syncitial virus among
others. According to the present invention, animal and
plant viral infections may also be treated by
administering the subject oligonucleotides.

The c-myc gene is one example of a gene which
25 can have a role in cell proliferation. Inhibition of c-myc
expression has been demonstrated in vitro using a
linear oligonucleotide complementary to a target 115 bp
upstream of the c-myc transcription start site (Cooney
et al., 1988, Science 241: 456-459). Circular
30 oligonucleotides of SEQ ID NO:1, and SEQ ID NO:2, as
depicted below, are complementary to the c-myc promoter
at nucleotides -131 to -120 and -75 to -62,

1 respectively, and are provided to inhibit c-myc expression in accordance with the present invention. As used in these depictions of SEQ ID NO:1 and SEQ ID NO:2, N can be any nucleotide or nucleotide analog.

5 SEQ ID NO:1

1
 N C T C C C C G C C C T C N
 N N N N
 N C T C C C C A C C C T C N

10

SEQ ID NO:2

1
N T C T T T T T C T T T T C N
N N N N
N T C T T T T T T C T T T T C N

Human immunodeficiency virus (HIV) is a
20 retrovirus causing acquired immunodeficiency syndrome
(AIDS). The circular oligonucleotides of this invention
provide a means of blocking the replication of the virus
without deleteriously affecting normal cellular
replication in humans infected with HIV. The retroviral
25 genome is transcribed as a single, long transcript, part
of which is spliced to yield RNA encoding viral envelope
proteins. Inhibition of HIV infection can be
accomplished by designing oligonucleotides to bind to a
number of regions within the HIV genome, including
30 coding regions for functions that replicate the genome
(i.e., the pol or reverse transcriptase function) or
functions that control gene expression (e.g. the tat,

35

1 rev or other functions). However, previous work with linear oligonucleotides has suggested that splice sites, poly(A) addition signals, cap or initiator codon sites, and sites implicated in ribosome assembly can be 5 particularly effective for inhibiting eucaryotic protein expression. Furthermore, the terminal structures of the retroviral genome are also excellent targets for inhibiting retrovirus production not only because these structures encode control regions which mediate the rate 10 of transcription and replication, but also because these structures are repeated, allowing an oligonucleotide to bind and block access to each repeat.

Accordingly, the present invention provides two circular oligonucleotides, set forth in SEQ ID NO:3 15 and SEQ ID NO:4 wherein N is any nucleotide or nucleotide analog and Y is a pyrimidine or a pyrimidine analog. SEQ ID NO:3 is complementary to an HIV-1 splice junction (nucleotides 6039-52), while SEQ ID NO:4 is complementary to part of the tat gene (nucleotides 5974- 20 88). The circular form of SEQ ID NO:3 is depicted below, wherein nucleotide number 1 is the first nucleotide in the P domain, i.e., the first T on the top line corresponds to base 1.

25

	1	
	N T T T C Y T C G T T C G T C N	
	N	N
	N	N
	N T T T C G T C A T T C A T C N	N

30 The circular form of SEQ ID NO:4 is depicted below wherein nucleotide number 1 is the first nucleotide of the P domain.

1

1

N T C C T T C T T C Y C C T C T N
N N N N
N T C C T T C T T C G C C T C T N

5

Circular oligonucleotides of SEQ ID NO:3 and SEQ ID NO:4 can inhibit HIV infection both in vitro and in vivo. In vitro screening for circular oligonucleotide effectiveness against HIV infection permits one skilled in the art to judge the stability of oligonucleotide: target binding and to assess in vivo efficacy and binding stability. To observe in vitro inhibition circular oligonucleotides can be added to the growth medium of an appropriate cell line infected with HIV. Cells can be pretreated with the circular oligonucleotides or circular oligonucleotides can be added at the time of infection or after HIV infection. Addition before or after infection allows assessment of whether the subject oligonucleotide can prevent or simply inhibit HIV infection respectively.

The extent of inhibition of HIV infection or replication can be judged by any of several assay systems, including assessment of the proportion of oligonucleotide-treated cells surviving after infection relative to survival of untreated cells, assessment of the number of syncytia formed in treated and untreated HIV infected cells and determination of the amount of viral antigen produced in treated and untreated cells.

In vivo studies of the efficacy of circular oligonucleotides can be done in a suitable animal host, such as transgenic mice, or chimpanzees. Levels of HIV

1 antigens can be monitored to assess the effect of circular oligonucleotides on HIV replication and thereby to follow the course of the disease state.

Alternatively, human volunteers with AIDS or ARC can be 5 administered with the subject circular oligonucleotides since the oligonucleotides do not appear to be cytotoxic. The disease status of these volunteers can then be assessed to determine the efficacy of the subject oligonucleotides in treating and preventing AIDS 10 infection.

A further aspect of this invention provides pharmaceutical compositions containing the subject circular oligonucleotides with a pharmaceutically acceptable carrier. In particular, the subject 15 oligonucleotides are provided in a therapeutically effective amount of about 0.1 μ g to about 100 mg per kg of body weight per day, and preferably of about 0.1 μ g to about 10 mg per kg of body weight per day, to bind to a nucleic acid in accordance with the methods of this 20 invention. Dosages can be readily determined by one of ordinary skill in the art and formulated into the subject pharmaceutical compositions.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion 25 media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is 30 incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary

1 active ingredients can also be incorporated into the compositions.

The subject oligonucleotides may be administered topically or parenterally by, for example, 5 intravenous, intramuscular, intraperitoneal subcutaneous or intradermal route, or when suitably protected, the subject oligonucleotides may be orally administered. The subject oligonucleotides may be incorporated into a cream, solution or suspension for 10 topical administration. For oral administration, oligonucleotides may be protected by enclosure in a gelatin capsule. Oligonucleotides may be incorporated into liposomes or liposomes modified with polyethylene glycol for parenteral administration. Incorporation of 15 additional substances into the liposome, for example, antibodies reactive against membrane proteins found on specific target cells, can help target the oligonucleotides to specific cell types.

Topical administration and parenteral 20 administration in a liposomal carrier is preferred.

The following examples further illustrate the invention.

25

30

35

1

EXAMPLE 1Circularization of Oligonucleotides Using an End Joining Oligonucleotide

5 According to the present invention, a simple one-step chemical method has been developed to construct circles from linear precursors (precircles). A DNA oligonucleotide was constructed which had the same sequence as the eventual target, this is the end-
10 joining-oligonucleotide. A precircle oligonucleotide was then constructed and chemically phosphorylated on the 5'-end or 3'-end. As depicted in Fig. 2, the precircle and end-joining-oligonucleotide were mixed and allowed to form a complex in which the ends were
15 adjacent. Cyanogen bromide, imidazole buffer, and a divalent metal were added. After incubation for 6-48 hr, the mixture was dialyzed, lyophilized, and the products were separated by denaturing 20% polyacrylamide gel electrophoresis. UV shadowing revealed major bands
20 which comigrated with the precircle and the end-joining-oligonucleotide, along with one new product which migrated slightly more slowly than the precircle. No product was observed without added end-joining-oligonucleotide or in the absence of a 5'- or 3'-
25 phosphate group on the precircle. The major bands were excised and eluted from the gel, dialyzed to remove salts and quantitated by absorbance at 260 nm. For reactions with precircles 1 and 2 (SEQ ID NO: 5 and SEQ ID NO: 6, respectively), using end-joining-
30 oligonucleotides 4 and 5 (SEQ ID NO: 8 and SEQ ID NO: 9, respectively), the circles 6 and 7 were obtained in 40% and 58% yields, respectively. The sequences of each of

1 these molecules and other oligonucleotides are depicted
in Fig. 3.

The circular structure of products 6 and 7 was confirmed by resistance to 3' exonuclease digestion and 5 to 5' dephosphorylation under reaction conditions in which a linear precircle was completely destroyed or dephosphorylated. Accordingly, the 3' exonuclease activity of T4 DNA polymerase cleaved linear precircles 1 and 2, but not circles 6 and 7. The linear precircles 10 were also 5'-end labeled with ^{32}P and then circularized. After reaction, the circular products were inert to calf alkaline phosphatase whereas the precircles completely released labeled ^{32}P . The slightly slower gel mobility of the circles relative to the precircles was consistent 15 with the occurrence of circularization.

Optimal Circularization Conditions

Many parameters were optimized to increase yields of the circular product, including oligonucleotide and precircle concentrations, 20 temperature, reaction time, metal, metal concentration, BrCN concentration and pH. Improved circularization conditions provided an at least two-fold higher yield of circles compared to prior art conditions wherein two single-stranded oligonucleotides were joined (Luebke 25 et al., 1989, J. Am. Chem. Soc. 111: 8733 and Kanaya et al., 1986, Biochemistry 25: 7423).

1 These improved conditions were:

50 μ M precircle
55 μ M end-joining-oligonucleotide
100 mM NiCl₂
5 200 mM imidazole HCl (pH 7.0)
125 mM BrCN
25°C, 36 hr.

However circle closure was also effective
under the following conditions:

10 3-200 μ M precircle
3-200 μ M end-joining-oligonucleotide
10-500 mM NiCl₂
50-500 mM imidazole-HCl
20-200 mM BrCN

15 4-37°C, 6-48 hr.

Other metals (Zn²⁺, Mn²⁺, Co²⁺, Cu²⁺, Pb²⁺,
Ca²⁺, Mg²⁺) also work in place of Ni²⁺. Additionally,
the reaction is pH sensitive.

Closure in AP and P Domains

20 Closure of a circle in the AP domain was
superior to closure in the P domain. Comparison of the
circularization of precircles 2 and 3 (SEQ ID NO: 6 and
SEQ ID NO: 7, respectively) around the same end-joining-
oligonucleotide (i.e. 5, SEQ ID NO: 9) indicated that
25 circle 7 (having SEQ ID NO: 6) was formed with a 58%
yield when closed in the AP domain (i.e. using precircle
2) and only a 35% yield when closed in the P domain
(i.e. using precircle 3).

Condensing Reagents

30 Two reagents have been commonly used for
chemical ligation of DNA and RNA, BrCN/imidazole/NiCl₂
and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)

1 (Kanaya *et al.* 1986 *Biochemistry* 25: 7423 and Ashley *et al.* 1991 *Biochemistry* 30: 2927). Therefore, these reagents were directly compared for efficacy in ligating a precircle to circular oligonucleotide 6 (Fig. 5 3 and SEQ ID NO: 5) using a dA₁₂ (SEQ ID NO: 8) end-joining-oligonucleotide.

BrCN/imidazole/NiCl₂ was used under the established optimal conditions except that ligation efficiency was observed at both 4°C and 25°C. EDC was 10 used at 200 mM with 20 mM MgCl₂, 50 mM MES (pH 6.0) at 4°C or 25°C with incubation for 4 days.

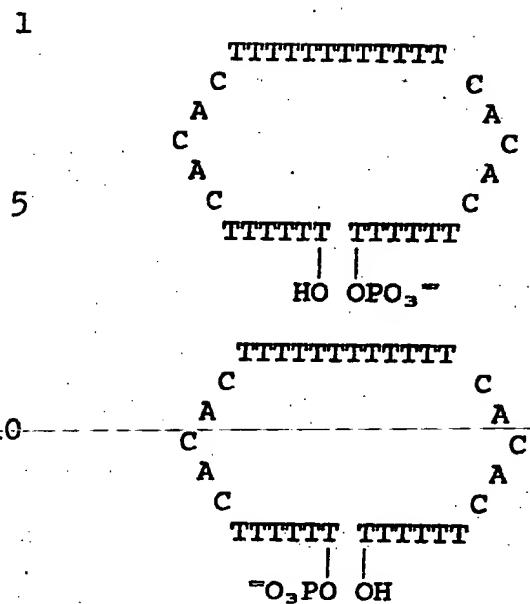
At 4°C BrCN was more efficient, yielding 95% circular product while EDC yielded only 55% product. However, at 25°C both EDC and BrCN yielded 95% product. 15 Therefore, BrCN is more effective at lower temperatures but either EDC or BrCN can be used with equal success at 25°C. However, BrCN has an additional advantage over EDC since ligation with BrCN requires 24 hr or less while ligation with EDC requires about 4 days.

20 Use of a 5'- or 3'-Phosphate

Under different ligation conditions joining a 3'-phosphate with a 5'-OH yielded more ligated product than joining a 5'-phosphate with a 3'-OH (Ashley, *et al.*).

25 Therefore, the percent conversion to circular oligonucleotide 6 (Fig. 3) by a 5'-phosphate or by a 3'-phosphate precircles was compared:

-56-



15 Circularization reactions were performed using
a dA₁₂ end-joining-oligonucleotide and the established
optimal conditions, except that 5 nmoles of precircle
and end-joining-oligonucleotide were used. Products
were visualized under UV light after separation by
20 denaturing gel electrophoresis.

Conversion to circular product was 60% ($\pm 5\%$) when a 5'-phosphate was present and 95% when a 3'-phosphate was present. No increase in yield was observed when increased reaction times or increased 25 reagent concentrations were used.

Accordingly, use of a 3'-phosphate rather than a 5'-phosphate improves circularization.

30

35

1

EXAMPLE 2Circular Oligonucleotid s Bind Target
Nucleic Acids with Higher Affinity
Than Do Linear Oligonucleotides

5 The binding affinities of circles 6 and 7 (SEQ ID NO: 5 and SEQ ID NO: 6, respectively) for their targets were measured by comparison of the melting temperatures of the circular and linear complexes.

10 Solutions contained 1:1 ratios of oligonucleotide and target (3 μ M each) in 100 mM NaCl, 10 mM MgCl₂, and 10 mM Tris-HCl (pH 7.0). Mixing curves measured at 260 nm confirmed that 1:1 complexes were formed. The free energies ($-\Delta G^{\circ}_{37}$) of the complexes were derived from the melting data using a two-state curve-fitting method (Petersheim, et al., 1983, Biochemistry 22: 256).

15 The results showed that the circular oligonucleotides bound to their targets more strongly than did linear precircles or Watson-Crick complementary target-sized oligonucleotides (Table 2). For example, target 4 (SEQ ID NO: 8) formed a duplex with its target-sized Watson-Crick complement having a T_m of 37.1°C while the precircle 1:target 4 complex (i.e. SEQ ID NO: 5 bound to SEQ ID NO: 8) had a T_m of 44.7°C. By comparison, circle 6, having the same sequence as precircle 1, bound to target 4 with a T_m of 57.5°C and a 20 free energy of binding that was 8.6 kcal/mol more favorable than the corresponding Watson-Crick duplex. The corresponding association constant at 37°C is $6 \times 10^{11} M^{-1}$, which is more than six orders of magnitude greater than for the Watson-Crick duplex. A similar 25 effect was observed for the binding of circle 7 (SEQ ID NO: 6) to target 5 (SEQ ID NO: 9); this complex had a T_m

1 of 62.3°C, whereas the corresponding Watson-Crick duplex melted at 43.8°C. These data indicate that the binding of circular oligonucleotides is stronger than the binding of a linear oligonucleotide to a target.

5 To determine the binding characteristics when the target sequence was embedded within a longer sequence, a 36 nucleotide oligonucleotide was synthesized with a 12 base target sequence (equivalent to target 4) in the middle. Melting studies revealed 10 that circle 6 bound to this longer oligonucleotide more strongly than it did to a target having the same size as the binding domains of the circle: the T_m of circle 6 with target 4 was 59.8°C whereas with the 36 base oligonucleotide containing an embedded target the T_m was 15 63.4°C. Therefore the binding strength of circles with embedded targets was higher than that with binding-domain-sized-targets.

The binding affinity of circle 6 for an RNA target was tested by synthesizing oligoribonucleotide 20 rA_{12} and determining the T_m of circle 6 with rA_{12} . The T_m of circle 6 with rA_{12} was 58.3°C compared with 57.8°C with dA_{12} . The data indicate that circles bind to RNA targets as strongly or more strongly than as to DNA targets.

25

30

35

-59-

1

TABLE II

5

oligonucleotide:

		target	complex	T _m , °C	- G° ₃₇ (kcal/mol)
		3' - TTTTTTTTTTT 5' - AAAAAAAA		37.1	8.1
10		3' - TTCTTTCTTC 5' - AAGAAAAGAAAG		43.8	10.3
15	1:4		TTTTTTTTTTTT C C A A C AAAAAAAA A C A A C C TTTTTT TTTTTT OPO ₃ ⁻	44.7	10.5
20	3:5		TTCTTTCTTC C C A A C AAGAAAAGAAAG C C A A C C TTCTTT TCTTTC OPO ₃ ⁻	47.0	10.8
25	6:4		TTTTTTTTTTTT C C A A C AAAAAAAA A C A A C C TTTTTTTTTTTT OPO ₃ ⁻	57.4	16.7
30	7:5		TTCTTTCTTC C C A A C AAGAAAAGAAAG C C A A C C TTCTTTCTTC	62.3	16.4
35					

-60-

1

EXAMPLE 3

Circular Oligonucleotides Bind Target More Selectively Than Linear Oligonucleotides

5 In order to measure the sequence selectivity of circular oligonucleotides, a set of target oligonucleotides with one variable base was constructed. Binding energies for a circle complexed with these targets were measured; the selectivity was defined by the 10 free energy difference between the correct sequence and mismatched sequences. The selectivity obtained with the circular structure was then directly compared to the selectivity of an analogous linear oligonucleotide.

DNA oligonucleotides were machine synthesized 15 using the β -cyanoethyl phosphoramidite method. Circular oligonucleotide 8 was prepared from a linear precircle having SEQ ID NO: 7:

5'-pTCTTCCACACCTTCTTCACACTTCTT

and was cyclized by assembly around an end-joining 20 oligonucleotide having the sequence 5'-AAGAAAAGAAAG (SEQ ID NO: 9) using BrCN/imidazole to close the final bond, as described in Example 1. The circular structure was confirmed by its resistance to a 3'-exonuclease and 5'- phosphatase.

25 The sequence selectivity of circle 8 was measured by hybridizing it with targets which contained a single mismatched base and determining the strength (ΔG°_{37}) of the resulting complexes by thermal denaturation. Eight targets were synthesized which were 30 complementary to circle 8 and linear oligonucleotide 9, except for a single centrally positioned variable base (X

-61-

1

or Y = A, G, C, T). Four targets have a variable base X which is matched with two opposing T's in the circle, resulting in a T-X-T triad. In the remaining four 5 targets, the variable base Y is matched with two opposing C's in the circle, giving a C-Y-C triad. For comparison to this circle complex, a linear oligonucleotide 9 was used; resulting in a duplex with a central T-X pair in the first four experiments or a C-Y pair in the remaining 10 four.

	<u>complex (X,Y = A,T,G,C)</u>	<u>expt. no.</u>
	3' - T T C T T T T C T T T C	
	5' - A A G A X A A G A A A G	1-4
15	A C T T C T T T T C T T T C C A	
	C A A G A X A A G A A A G C	5-8
	A C T T C T T T T C T T T C C A	
20	3' - T T C T T T T C T T T C	
	5' - A A G A A A A Y A A A G	9-12
	A C T T C T T T T C T T T C C A	
25	C A A G A A A A Y A A A G C	13-16
	A C T T C T T T T C T T T C C A	

Thermal denaturation of the sixteen complexes was carried out in the presence of 10 mM MgCl₂, 100 mM 30 NaCl, and 10 mM Tris-HCl (pH 7.0), with target and circular or linear oligonucleotide concentrations at 3 μ M

-62-

1

each. Experiments were carried out in duplicate and the results averaged. Oligonucleotide:target complex melting was monitored at 260 nm. The temperature vs. absorbance 5 curves so generated showed a single transition from bound to free oligonucleotide. Free energies of association were obtained by fitting the data with a two-state curve-fitting method. The results were checked in two cases by measuring the association energies by the van't Hoff 10 method, good agreement was seen between the two methods. Selectivities are defined as the difference in free energies (ΔG) of complexation between matched and mismatched oligomers.

15 Table III displays the results of the mismatch experiments. Experiments 1-4 show the effects of a T-X target mismatch on a DNA duplex. As expected, the true match (X = A) gives the most favorable complex ($-\Delta G^\circ_{37} = 10.3$ kcal/mol); the mismatches (X = G, C, T) result in a loss of 3.2-4.4 kcal/mol in binding energy, in good 20 agreement with published mismatch studies. Experiments 5-8, by comparison, show the effects of a T-X-T mismatch on circle complex binding strength. Once again, the true match (X = A) gives the most favorable three stranded complexes ($-\Delta G^\circ_{37} = 16.4$ kcal). However, target 25 mismatches (X = G, T, C) result in a considerably larger loss of binding energy (6.2-7.6 kcal/mol) for a circular oligonucleotide than for a linear oligonucleotide.

Similarly, experiments 9-12 give the effects of a C-Y mismatch on the two stranded duplex. The matched 30 base (Y = G) gives a free energy of duplex association of -10.3 kcal/mol. The mismatches (Y = A, T, C) result in a

-63-

1

loss of 5.2 to 5.8 kcal/mol of binding energy, in reasonable agreement with published data. By contrast, the effects of a C-Y-C mismatch are greater in a three 5 stranded complex (experiments 13-16): the match (Y = G) gives a binding energy of -16.4 kcal/mol, and the mismatches (Y = A, T, C) are less stable by 7.1 to 7.5 kcal/mol.

Thus, in all the cases studied, the circular 10 ligand shows greater selectivity for its correctly matched sequence than does the standard linear oligomer. The selectivity advantage ranges from 1.3 to 2.2 kcal/mol for the C-Y-C series to 3.0 to 3.4 kcal/mol for the T-X-T series. These are quite significant differences, 15 considering they arise from a single base change; in the T-X-T series, the circular oligonucleotide is nearly twice as selective as the linear oligonucleotide. This selectivity difference corresponds to one to two orders of magnitude in binding constant at 37°C.

20 There are two factors which may explain this high selectivity. First, because two domains of the circular oligonucleotide bind the central target strand, the circular oligonucleotide, in effect, checks the sequence twice for correct matching. Secondly,

25 protonation of cytosine within a C+G-C triad may also be a factor in increasing selectivity. This protonation is likely to be favored only when there is base triad formation wherein guanine can share the positive charge; evidence suggests that the pKa of cytosine within a base 30 triad is 2-3 units higher than that of free deoxycytosine. The addition of this positive charge may

-64-

1

lessen the negative charge repulsions arising from the high density of phosphates in the complex and thereby increase binding stability.

5 Therefore, circular oligonucleotides, as described herein, to have both higher binding affinity and higher selectivity than can be achieved with Watson-Crick duplexes alone.

10

15

20

25

30

35

-65-

1

TABLE III

5	expt. #	variable base	T _m , °C	-ΔG° ₃₇ (kcal/mol)	Selectivity (kcal/mol)
10 duplex	1	X=A	43.8	10.3	--
	2	X=G	33.8	7.1	3.2
	3	X=C	28.3	5.9	4.4
	4	X=T	31.1	6.4	3.9
15 circle complex	5	X=A	62.3	16.4	--
	6	X=G	44.2	10.2	6.2
	7	X=C	39.8	8.8	7.6
	8	X=T	40.8	9.1	7.3
	9	Y=A	26.2	5.1	5.2
20 duplex	10	Y=G	43.8	10.3	--
	11	Y=C	22.2	4.5	5.8
	12	Y=T	27.0	5.0	5.3
	13	Y=A	39.9	9.0	7.4
25 circle complex	14	Y=G	62.3	16.4	--
	15	Y=C	41.3	9.3	7.1
	16	Y=T	39.6	8.9	7.5

30

35

-66-

1

EXAMPLE 4

Factors Effecting Complex Formation

1) Solution effects. The effects of NaCl, 5 Mg^{2+} , spermine, and pH on circle:target complexes were examined. Circles with cytosines in the binding domains are sensitive to pH, and exhibited greater stability at lower pH values. However, these and other circle:target complexes are quite stable at the physiological pH of 10 7.0-7.4 (Fig. 5). The complexes show salt concentration sensitivity comparable to duplexes; however, small amounts of Mg^{2+} or spermine increase the complex stability markedly. For example, in a concentration of 1 mM Mg^{++} at pH 7.0, with no added salts, a stable 7:5 15 circle:target complex formed having a T_m of 58°C. When a solution of 20 μM spermine containing no added salts was used the 7:5 complex again formed stably with a T_m of 56°C. Both Mg^{++} and spermine are present in at least these concentrations in mammals, and so circle:target 20 complexes will be stable under physiological conditions.

2) Loop size. The optimum number of nucleotides for the loop domain of a circle was determined by observing complex formation between a target and circles with different loop sizes. Precircle 25 linear oligonucleotides similar to precircle 1 were synthesized with 2, 3, 4, 5, 6 and 10 base loops using an arbitrary sequence of alternating C and A residues. Each of these precircles was designed to bind to the A_{12} template (i.e. target 4 (SEQ ID NO: 8)). The T_m 's for 30 circles with 4, 5, 6 and 10 base loops showed that a five-nucleotide loop size was optimum for the circle

-67-

1

binding either to template A₁₂ or to a longer 36mer sequence containing the A₁₂ binding site (see Fig. 6A).

5 3) Binding Domain length. The effect of circular oligonucleotide binding domain length on circle:target complex melting temperature was compared to melting of duplexes having the same length. Circles with various size binding domains were constructed and complexed with single-stranded dA_n targets for n equal to 10 4, 8, 12 and 18 nucleotides. Fig. 6B illustrates that considerably higher T_m's were observed for circle:target complexes relative to Watson-Crick duplexes having the same length as the binding domains (determined in 0.1 M NaCl, pH 7). For example, a 12-base circular complex 15 melted at about the same temperature as a 24-base duplex. The 4-base circular complex melted at 34°C, whereas the corresponding Watson-Crick duplex T_m was less than 0°C.

20 4) Methylation. It has been known for some time that methylation at the C-5 position of cytosine, forming the naturally-occurring base m⁵C, raises the T_m of duplex DNA in which it occurs, relative to unmethylated sequences (Zmudzka et al., 1969, Biochemistry 8: 3049). In order to investigate whether addition of this methyl group would stabilize 25 circle:target complexes, two analogs of circle 7 (having SEQ ID NO: 6) were synthesized. In one circle, the six C's in the binding domains were methylated leaving the loop unmethylated (Me₆). In the second circle, all twelve C's were methylated (Me₁₂). Melting temperatures 30 for the complexes of these methylated circle with target 5 were measured. The Me₆ complex had a T_m of 71.1°C

-68-

1

(compared to 61.8°C for the unmethylated circle), and the Me₁₂ circle had a T_m of 72.4°C. Thus, use of the natural base m⁵C in place of C increased stability substantially, and in one case resulted in a 12-base complex which melted 10.6°C higher than an unmethylated circle and 28.6°C higher than the corresponding unmethylated Watson-Crick duplex.

10

15

20

25

30

35

-69-

1

EXAMPLE 5

Replacement of Nucleotide Loop Domains
with Non-Nucleotide Loop Domains

5

The loop domains of circular oligonucleotides were replaced with polyethylene or oligoethylene glycol chains of different lengths and the effect of such synthetic loops upon circular oligonucleotide binding and nuclease resistance was assessed.

10

Methods

Circular oligonucleotides were synthesized having tetra-, penta-, or hexa-ethylene glycol chain loop domains. In each case the ethylene glycol chain was synthetically prepared for automated DNA synthetic 15 procedures using the method of Durand *et al.* (1990, Nucleic Acids Res. 18: 6353-6359). Briefly, a phosphoramidite was placed on a hydroxy group at one end of the ethylene glycol chain and a dimethoxytrityl (DMT) moiety was placed on the other terminal ethylene glycol 20 hydroxy group. This derivatized ethylene glycol chain was then added to the growing linear oligonucleotide at the appropriate step of automated DNA synthesis. Circularization steps were performed by procedures described in Example 1. A linear oligonucleotide 25 precircle having a tetraethylene loop domain was not efficiently circularized. This result indicates that a tetraethylene loop domain may be too short for optimal binding to a target.

Two types of linear oligonucleotides were used 30 as target binding domains for the circular oligonucleotides: Target I was a 12-base oligonucleotide

-70-

1

having no non-target nucleotides and Target II was a 36-base oligonucleotide having a 12-base target within it.

The target sequences utilized were 5'-AAGAAAAGAAAG-3'

5 (SEQ ID NO: 9) and 5'-AAAAAAAAAAA-3' (SEQ ID NO: 8), the latter is termed a poly(dA)₁₂ target sequence.

The melting temperatures (T_m) of circular oligonucleotides with polyethylene loops were observed at pH 7.0 (10 mM Tris-HCl) in 10 mM MgCl₂ and 100 mM NaCl.

10 Each linear target and each circular oligonucleotide was present at a 3 μ M concentration.

Results

15 The T_m of a circular oligonucleotide having a CACAC nucleotide loop sequence and a poly(dT)₁₂ sequence for both P and AP domains was 57.8 °C when bound to a poly (dA)₁₂ target sequence. The T_m of a circular oligonucleotide having the same P and AP domain sequences but hexaethylene glycol loop domains was 51.4 °C when bound to the same target.

20 A comparison of T_m values observed for circular oligonucleotides having pentaethylene glycol (PEG) and hexaethylene glycol (HEG) loop domains is depicted in Table IV.

25

30

35

-71-

1

TABLE IV

5

Complex

Target Target
I Tm II Tm

10

p T T C T T T C T T T C p
 PEG A A G A A A A G A A A G PEG
 p T T C T T T C T T T C p

51.5 47.5

15

p T T C T T T C T T T C p
 HEG A A G A A A A G A A A G HEG
 p T T C T T T C T T T C p

58.0 51.1

20

p T T T T T T T T T T p
 HEG A A A A A A A A A A HEG
 p T T T T T T T T T T p

51.4 46.5

The T_m value observed for a circular oligonucleotide having a HEG loop is about 4.5°C higher than that of a circular oligonucleotide with a PEG loop. Therefore, circular oligonucleotides with hexaethylene glycol loop domains bind with greater stability than do circular oligonucleotides with tetra- or penta-ethylene glycol loops.

30

35

-72-

1

Nuclease Resistance

5 Circular oligonucleotides were tested for nuclease resistance when unbound and when bound to a target oligonucleotide. All circular oligonucleotides, whether bound or unbound, were completely resistant to exonucleases. Endonuclease sensitivity was assessed using S1 nuclease according to the manufacturer's suggestions.

10 A comparison of the resistance of bound and unbound circular oligonucleotides to S1 nuclease is depicted in Table V.

15

20

25

30

35

-73-

1

TABLE V

5

Oligonucleotide
CleavageTime For
50% S1

10

p T T C T T T T C T T T C p

HEG

HEG

1 min.

p T T C T T T T C T T T C p

15

p T T C T T T T C T T T C p
HEG A A G A A A A G A A A G HEG
p T T C T T T T C T T T C p

> 24 h

20

A C T T C T T T T C T T T C C A

C

C

1 min.

A C T T C T T T T C T T T C C A

A C T T C T T T T C T T T C C A
C A A G A A A A G A A A G C
A C T T C T T T T C T T T C C A

40 min.

25

30

35

-74-

1

These data indicate that unbound circular oligonucleotides are vulnerable to S1 nuclease. However, when bound to a target, a circular oligonucleotide having 5 a polyethylene loop domain is much more resistant to S1 nuclease, at least 36-fold more resistant, than a circular oligonucleotide with a nucleotide loop domain.

The nuclease resistance of circular and linear oligonucleotides was also compared when these 10 oligonucleotides were incubated in human plasma for varying time periods. Circular oligonucleotide 7 and the precursor to this circle, linear oligonucleotide 2, were incubated at a 50 μ M concentration in plasma at 37°C. Aliquots were removed at various time points and cleavage 15 products were separated by gel electrophoresis. Nuclease resistance was assessed by observing whether degradation products were evident on the gels.

When incubated in human plasma the half-life of linear oligonucleotide 2 was 20 min. In contrast, 20 circular oligonucleotide 7 underwent no measurable nuclease degradation during a 48 hr incubation. Accordingly, the half-life of a circular oligonucleotide is greater than 48 hr in human plasma, i.e. more than 140 times longer than a linear oligonucleotide having an 25 equivalent sequence.

30

35

-75-

1

EXAMPLE 6
CIRCULAR OLIGONUCLEOTIDES CAN SELECTIVELY
BIND TO RNA

5

Experiments described in this Example indicate that, unlike linear oligonucleotides, circular oligonucleotides can preferentially bind to an RNA, rather than a DNA, target.

10

Two linear deoxyoligonucleotides were prepared as targets, a "T" (SEQ ID NO.: 11) target and a "dU" (SEQ. ID. No.: 12) target:

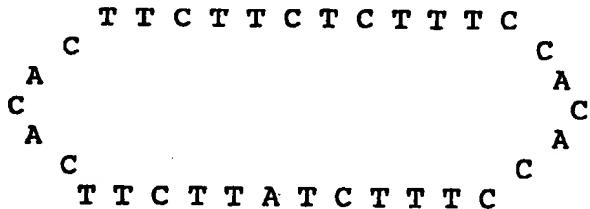
T target: 5'-A A G A A T A G A A A G-3'; and

15

dU target: 5'-A A G A A U A G A A A G-3'.

A circular oligonucleotide having SEQ ID NO.: 14 was also prepared:

20



For comparison, a linear oligonucleotide complementary to 25 the T and dU targets was also synthesized (i.e. the linear oligonucleotide, SEQ ID NO.: 13):

5'C T T T C T A T T C T T 3'.

The melting temperatures (T_m) values observed for the circular vs linear oligonucleotide binding to 30 each of the targets is presented in Table VI.

-76-

1

TABLE VI

5

T_m Values for Oligonucleotides

10

Targets	Linear	Circular
T target	42.9° C	41.1° C
dU target	40.9° C	42.9° C

15 The linear oligonucleotide binds more strongly to the T target than to the dU target, by an amount which is significantly larger than experimental error limits.

15 This difference in T_m values corresponds to a difference in free energy of binding of 1.7 kcal/mole.

20 However, in contrast to the linear oligonucleotide, the circular oligonucleotide binds more strongly to the U target. Therefore, the circular oligonucleotide can exhibit a preference for an RNA target relative to the corresponding DNA target.

25 Moreover, the increase in binding strength for a circular oligonucleotide to the RNA target corresponds to a free energy difference of 0.8 kcal/mole which indicates that at 37 °C an RNA target would be preferred by about 3:1 over a corresponding DNA target.

30

35

1

EXAMPLE 7

Strand Replacement By Circular Oligonucleotides

5 Circular oligonucleotide 6 (Fig. 3) bound to a
dA₁₂ target with 9 kcal/mole greater stability than did a
linear dT₁₂ oligonucleotide (Example 2). This increase
in stability demonstrates that a circular-
oligonucleotide:target complex is thermodynamically
10 favored over a linear-oligonucleotide:target. In
addition, a circular oligonucleotide can actually
accelerate (or catalyze) dissociation of duplex DNA
target sequences to form a complex with one strand of the
duplex.

15 To test whether a circular oligonucleotide can readily dissociate duplex DNA and displace one strand of a duplex DNA target, the kinetics of strand displacement were observed for a duplex DNA target in the presence of a complementary linear or circular oligonucleotide.

20 A DNA duplex target with a fluorescein group on
one strand and a tetramethylrhodamine group on the other
strand was prepared using published procedures (Cardullo
et al. 1988 Proc. Natl. Acad. Sci. USA 85: 8790; Cooper
et al. 1990 Biochemistry 29: 9261). The structure of the
25 duplex target (SEQ ID NO.: 15) was as follows:

25 duplex target (SEQ ID NO.: 15) was as follows:

5'-fluorescein-A A A A A A A A A A A A A
3'-rhodamine-T T T T T T T T T T T T T.

The T_m of this labeled duplex target was normal, therefore the fluorescent substituents had no significant effect upon association kinetics. Moreover, the emission maxima of the fluorescein-dA_{1,2} strand was 523 nm while the

-78-

1

emission maxima of the rhodamine-dT₁₂ strand was 590 nm, allowing the association kinetics of the two strands could be separately monitored.

5

Strand displacement reactions were done at 10°C in a 1 cm fluorescence cuvette. Reaction conditions were 100 mM NaCl, 10 mM Mg Cl₂ and 10 mM Tris-HCl, pH 7.0 with a reaction volume of 3 ml. Labeled duplex was allowed to equilibrate for at least 1 hr at 10°C before addition of

10

a 40-fold excess of linear or circular oligonucleotide (final concentration 0.01 μM). A Spex Flurolog F 111A fluorescence instrument with 5 mm slit widths was used.

15

An excitation wavelength of 450 nm and a monitored emission wavelength of 523 nm was used. The results were independent of both excitation and monitored emission wavelengths. Reactions were followed for at least 5 half-lives.

20

Addition of rhodamine-dT₁₂ to fluorescein-dA₁₂ caused a decrease in fluorescein fluorescence and an increase in rhodamine fluorescence. Such effects are due to energy transfer between the fluorescent moieties (Cardullo *et al.*).

25

The association rate constant of the two fluorescently-labeled strands was determined by mixing the strands under pseudo-first order conditions and monitoring the rate of decrease in fluorescein emission. At 10 °C the observed association constant was 3.2×10^6 M⁻¹ sec⁻¹, which agrees well with published rates of association for DNA oligonucleotides (Nelson *et al.* 1982, Biochemistry 21: 5289; Turner *et al.* 1990 in Nucleic

-79-

1

Acids (subvolume C), W. Saenger, Ed. Springer-Verlag, Berlin: 201-227).

5 To compare the rates at which a single linear strand (SEQ ID NO.: 8) or a circular oligonucleotide having SEQ ID NO.: 5 (i.e. circular oligonucleotide 6) exchanged with strands in a duplex DNA, an excess of an unlabeled linear or circular oligonucleotide was mixed with the fluorescently-labeled duplex DNA target. The 10 increase in fluorescein emmission was then observed at a temperature significantly below the T_m of the duplex target as a measure of duplex target strand dissociation.

Fig. 8 depicts a typical kinetic assay for the dissociation of duplex target by a 40-fold excess of 15 unlabeled dA₁₂ (dotted line) or circular oligonucleotide 6 (solid line) at 10 °C. As depicted, duplex target dissociation by the circular oligonucleotide is considerably faster than is the dissociation by the linear oligonucleotide. The first order rate constant 20 for dissociation by the linear oligonucleotide is 2.0 x 10⁻⁴ sec⁻¹ whereas the first order rate constant for dissociation by the circular oligonucleotide is 2.3 x 10⁻² sec⁻¹, almost two orders of magnitude faster. This difference is even more apparent when the half-lives for 25 the target duplex in the presence of linear vs circular oligonucleotides are calculated. At 10 °C, the duplex has a half-life for dissociation of 58 min in the presence of the linear oligonucleotide but only 30 sec in the presence of the circular oligonucleotide.

30 Unlike the rate of reaction between linear oligonucleotide and duplex, the rate of reaction between

-80-

1

the circular oligonucleotide and duplex is dependent on the concentration of added circular oligonucleotide at low concentrations, and shows Michaelis-Menten type 5 saturation behavior at higher concentrations (Fig. 9).

The dissociation rate of labeled duplex at 10°C can be derived from the duplex association rate constant and $\Delta G^{\circ}_{1,0}$ values. This rate constant, $8.5 \times 10^{-10} \text{ sec}^{-1}$, is consistent with rates derived from predicted 10 thermodynamic parameters for a duplex complex (Breslauer et al. 1986 Proc. Natl. Acad. Sci. USA 83: 3746) although this rate is significantly slower than the rate constant for strand displacement by a linear oligonucleotide. An increase in duplex dissociation upon addition of a linear 15 oligonucleotide has been noted in other cases (Chamberlin et al. 1965 J. Mol. Biol. 12: 410). Comparison of the rate for the circular oligonucleotide-catalyzed reaction over that of the unassisted duplex dissociation reveals a rate enhancement of about 10⁷ fold (Sigler et al. 1962 J. 20 Mol. Biol. 5: 709).

A double reciprocal plot of $1/[\text{circular oligonucleotide}]$ vs. $1/k_{\text{obs}}$ is linear and yields a k_{cat} of $0.024 \pm 0.005 \text{ sec}^{-1}$ and a K_m of $2.2 \times 10^{-7} \text{ M}$. The k_{cat} is 100-fold greater than the observed rate constant 25 obtained for the reaction of the duplex with either dA₁₂ or dT₁₂ single strands.

The observed saturation behavior (Fig. 9) suggests that a complex forms between the circle and the double-stranded target. Using the above K_m value and 30 assuming that $k_{\text{cat}} \ll k_{-1}$, where k_{-1} is the dissociation rate constant for this complex, the free energy of

-81-

1

association is $-8.6 \text{ kcal}\cdot\text{mol}^{-1}$ at 10°C . This value is similar to an estimated value of about $-9 \text{ kcal}\cdot\text{mol}^{-1}$ for the P domain in a 12-base triple helix consisting of T·A-T base triads, as derived from the thermodynamic parameters of Pilch et al. (1990 Nucleic Acids Res. 18: 5743).

10

15

20

25

30

35

-82-

1

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Kool, Eric T.

(ii) TITLE OF INVENTION: SINGLE-STRANDED, CIRCULAR
OLIGONUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 15

10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Scully, Scott, Murphy & Presser
(B) STREET: 400 Garden City Plaza
(C) CITY: Garden City
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 11530

15

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version

#1.25

20

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

25

(A) NAME: McNulty, William E.
(B) REGISTRATION NUMBER: 22,606
(C) REFERENCE/DOCKET NUMBER: 8085Z

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (516) 742-4343
(B) TELEFAX: (516) 742-4366

30 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

35

-83-

1

(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCCCCGCC C TCNNNNNCTC CCACCCCTCN NNNN

34

10 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTTTTTCT T T T C N N N N C T T T C T T T T T T C T N N N N N

38

20 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

T T T C Y T C G T T C G T C N N N N N C T A C T T A C T G C T T T N N N N N

38

30 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

35

-84-

1

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: circular

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCCTTCTTCY CCTCTNNNN TCTCCGCTTC TTCCTNNNN

40

10 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTTTCACA CTTTTTTTTT TTTCACACTT TTTT

34

20 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCTTTCCACA CCTTTCTTTT CTTCACACTT CTTT

34

30 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

35

-85-

1

(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: both

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTTCITCACA CTTCTTTCT TTCCACACCT TTCT
34

10 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAAAAAAAAA AA
12

20 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAGAAAAGAA AG
12

30 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

35

-86-

1

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTTTCTTTC TT
12

10 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGAATAGAA AG
12

20 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAGAAUAGAA AG
12

30 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

35

-87-

1

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTTTCTATTCTT TT
12

10 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTCTTCTCTT TCCACACCTT TCTATTCTTC ACAC
34

20 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAAAAAAAAA AA
12

30

35

-88-

1

WHAT IS CLAIMED:

1. A single-stranded circular oligonucleotide comprising at least one parallel binding (P) domain and 5 at least one anti-parallel binding (AP) domain having a loop domain between each binding domain to form said circular oligonucleotide; each P and corresponding AP domain having sufficient complementarity to bind detectably to one strand of a defined nucleic acid target 10 wherein said P domain binds in a parallel manner to said target, and said corresponding AP domain binds in an anti-parallel manner to said target.

2. The oligonucleotide of Claim 1 wherein said target comprises a known nucleotide sequence from which a 15 nucleotide sequence for a sufficient number of positions in said P domain and in said corresponding AP domain is determined from the sequence of said target for said P domain:

when a base for a position in said target is 20 guanine or a guanine analog, then P has cytosine, or a suitable analog thereof, in a corresponding position;

when a base for a position in said target is adenine, or an adenine analog then P has thymine or uracil, or suitable analogs thereof, in a corresponding 25 position;

when a base for a position in said target is thymine, or a thymine analog, then P has cytosine or guanine, or suitable analogs thereof, in a corresponding position;

30 when a base for a position in said target is cytosine, or a cytosine analog, then P has cytosine,

-89-

1

thymin or uracil, or suitable analogs thereof, in a corresponding position; and

5 when a base for a position in said target is uracil, or a uracil analog, then P has cytosine, guanine, thymine or uracil, or suitable analogs thereof, in a corresponding position;

and for said AP domain:

10 when a base for a position in said target is guanine, or a guanine analog, then AP has cytosine or uracil, or suitable analogs thereof, in a corresponding position;

15 when a base for a position in said target is adenine, or an adenine analog, then AP has thymine or uracil, or suitable analogs thereof, in a corresponding position;

20 when a base for a position in said target is thymine, or a thymine analog, then AP has adenine, or a suitable analog thereof, in a corresponding position;

when a base for a position in said target is cytosine, or a cytosine analog, then AP has a guanine, or a suitable analog thereof, in corresponding position; and

25 when a base for a position in said target is uracil, or a uracil analog, then AP has adenine or guanine, or suitable analogs thereof, in a corresponding position;

30 wherein said sufficient number of positions is that number of positions to provide sufficient complementarity for said oligonucleotide to bind detectably to said target.

-90-

1

3. The oligonucleotide of Claim 1 wherein said P domain comprises a nucleotide sequence which is determined from a known nucleotide sequence of said 5 target:

when a base for a position in said target is guanine or a guanine analog, then P has cytosine, or a suitable analog thereof, in a corresponding position;

10 when a base for a position in said target is adenine, or an adenine analog then P has thymine or uracil, or suitable analogs thereof, in a corresponding position;

15 when a base for a position in said target is thymine, or a thymine analog, then P has cytosine or guanine, or suitable analogs thereof, in a corresponding position;

20 when a base for a position in said target is cytosine, or a cytosine analog, then P has cytosine, thymine or uracil, or suitable analogs thereof, in a corresponding position;

when a base for a position in said target is uracil, or a uracil analog, then P has cytosine, guanine, thymine or uracil, or suitable analogs thereof, in a corresponding position;

25 and further wherein said AP domain comprises a nucleotide sequence which is determined from said sequence of said target as follows:

30 when a base for a position in said target is guanine, or a guanine analog, then AP has cytosine or uracil, or suitable analogs thereof, in a corresponding position;

-91-

1

when a base for a position in said target is adenine, or an adenine analog, then AP has thymine or uracil, or suitable analogs thereof, in a corresponding 5 position;

when a base for a position in said target is thymine, or a thymine analog, then AP has adenine, or a suitable analog thereof, in a corresponding position;

when a base for a position in said target is 10 cytosine, or a cytosine analog, then AP has a guanine, or a suitable analog thereof, in corresponding position; and

when a base for a position in said target is uracil, or a uracil analog, then AP has adenine or guanine, or suitable analogs thereof, in a corresponding 15 position.

4. The oligonucleotide of Claim 1, 2 or 3 wherein said target, said P domain and said AP domain independently comprise from about 2 to about 200 nucleotides.

20 5. The oligonucleotide of Claim 4 wherein said target, said P domain and said AP domain independently comprise from about 6 to about 36 nucleotides.

6. The oligonucleotide of Claim 1, 2 or 3 wherein each loop domain independently comprises from 25 about 2 to about 2000 nucleotides.

7. The oligonucleotide of Claim 6 wherein each loop domain independently comprises from about 3 to about 8 nucleotides.

8. The oligonucleotide of Claim 1, 2 or 3 30 wherein said target is single stranded or double stranded.

-92-

1

9. The oligonucleotide of Claim 1, 2 or 3 wherein said target is RNA or DNA.

5 10. The oligonucleotide of Claim 1, 2 or 3 wherein said target is a domain contained in a nucleic acid template.

11. The oligonucleotide of Claim 1, 2 or 3 wherein said P domain and said AP domain bind to said target in a staggered binding arrangement.

10 12. The oligonucleotide of Claim 1 or 2 wherein sufficient complementarity is less than 100% complementarity.

15 13. The oligonucleotide of Claim 12 wherein sufficient complementarity is about 30% to about 40% complementarity.

14. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide is DNA or RNA.

20 15. The oligonucleotide of Claim 2 or 3 wherein a suitable analog of cytosine is 5-methylcytosine.

16. The oligonucleotide of Claim 2 or 3 wherein a suitable analog of uracil is 5-methyluracil.

17. The oligonucleotide of Claim 2 or 3 wherein a suitable analog of adenine is diaminopurine.

25 18. The oligonucleotide of Claim 1, 2 or 3 wherein nucleotides have a 2'-O-methylribose in place of ribose or deoxyribose.

19. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide is taken up in a cell.

30 20. The oligonucleotide of Claim 19, wherein said oligonucleotide further comprises a ligand for a

-93-

1

cellular receptor, cholesterol group, an aryl group, a steroid group or a polycation.

5 21. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide further comprises a drug or a drug analog.

22. The oligonucleotide of Claim 1, 2 or 3 wherein said loop domains comprise non-nucleotide loop domains.

10 23. The oligonucleotide of Claim 22 wherein said non-nucleotide loop domains are polyethylene glycol.

24. The oligonucleotide of Claim 23 wherein said polyethylene glycol is pentaethylene glycol, hexaethylene glycol or heptaethylene glycol.

15 25. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide further comprises at least one methylphosphonate, phosphorothioate, phosphorodithioate, phosphotriester, siloxane, carbonate, acetamide, thioether or phosphorus-boron linkage.

20 26. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide further comprises a reporter molecule.

27. A compartmentalized kit for detection or diagnosis of a target nucleic acid, comprising:

25 ~~- at least one first container providing a circular oligonucleotide of any one of Claims 1-3.~~

28. A compartmentalized kit for isolation of a template nucleic acid, comprising at least one first container providing a circular oligonucleotide of Claim 1, 2 or 3, wherein said oligonucleotide is complementary to a target contained within said template.

-94-

1

29. The kit of Claim 28 wherein said template is poly (A)⁺ mRNA.

5 30. A method of regulating biosynthesis of a DNA, an RNA or a protein which comprises:

10 contacting at least one oligonucleotide of any one of Claims 1 to 3 with a nucleic acid template for said DNA, said RNA or said protein, under conditions sufficient to permit binding of said at least one oligonucleotide to a target sequence contained within said template;

15 binding said oligonucleotide to said target; blocking access to or allowing degradation of said template and thereby regulating biosynthesis of said DNA, said RNA or said protein.

20 31. The method of Claim 30 wherein said template comprises a double-stranded nucleic acid target, and wherein said conditions are effective to denature said target by strand displacement and thereby permit binding.

25 32. The method of Claim 30 wherein said biosynthesis comprises at least one of DNA replication, DNA reverse transcription, RNA transcription, RNA splicing, RNA polyadenylation, RNA translocation and protein translation.

33. The method of Claim 32 wherein said template for said DNA replication is an RNA template or a DNA template.

30 34. The method of Claim 33 wherein said target of said oligonucleotide for regulating said DNA

-95-

1

replication is an origin of replication or a primer binding site.

5 35. The method of Claim 32 wherein said target of said oligonucleotide for regulating said DNA reverse transcription is a primer binding site, a site in a retroviral genome, or a site in an mRNA.

10 36. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA transcription is a promoter, a repressor binding site, an operator, an enhancer, a transcription regulatory element or a site in an mRNA encoding region.

15 37. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA splicing is at least one of a 5' splice junction, an intron branch point or a 3' splice junction.

20 38. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA polyadenylation is a polyadenylation site.

25 39. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA translocation is a poly(A) tail.

40. The method of Claim 32 wherein said template for said protein translation is an mRNA template.

41. The method of Claim 40 wherein said target of said template is a ribosome binding site, a 5' mRNA cap or a site in a protein coding region.

30 42. The method of Claim 30 wherein said template is a viral DNA or RNA template.

-96-

1

43. The method of Claim 42 wherein said oligonucleotide has a nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

5

44. A method of strand displacement in a double-stranded nucleic acid target which comprises contacting said target with a circular oligonucleotide of any one of Claims 1-3 for a time and under conditions effective to denature said target and to 10 bind said circular oligonucleotide.

10

45. The method of Claim 44 wherein said conditions effective to denature said target comprise having said circular oligonucleotide and said target present in a ratio of about 1 to about 100.

15

46. The method of Claim 45 wherein said time effective to denature said target ranges from about 1 minute to about 16 hours.

20

47. The method of Claim 44 wherein said double-stranded nucleic acid target comprises a viral, a bacterial, a fungal or a mammalian nucleic acid.

25

48. The method of Claim 47 wherein said double-stranded nucleic acid target is an origin of replication, a promoter, a repressor binding site, an operator, an enhancer, a transcription regulatory element or a site in an mRNA encoding region.

30

49. The method of Claim 44 wherein said double-stranded nucleic acid target is present in a pure or impure nucleic acid sample, a tissue section, a cell smear or a chromosomal squash.

35

-97-

1

50. The method of Claim 49 wherein said oligonucleotide is covalently linked to a reporter molecule.

5

51. A pharmaceutical composition for regulating biosynthesis of a nucleic acid or protein comprising a biosynthesis regulating amount of at least one of the oligonucleotides of Claims 1 to 3 and a pharmaceutically acceptable carrier.

10

52. A method of preparing the single-stranded circular oligonucleotide of Claim 1, 2 or 3 comprising binding a linear precircle-to-an-end-joining- oligonucleotide, joining two ends of said precircle and recovering said single-stranded circular oligonucleotide.

15

53. The method of Claim 52 wherein said linear precircle has a 3'-phosphate.

20

54. The method of Claim 53 wherein said two ends comprise two nucleotides corresponding to AP nucleotides of said single-stranded circular oligonucleotide.

25

55. The method of Claim 54 wherein said joining is performed with BrCN, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide or N-cyanoimidazole ZnCl₂.

25

56. A complex formed between the oligonucleotide of Claim 1, 2 or 3 and a target.

57. A method of specific cell type drug delivery comprising:

30

a) administering to an animal a drug covalently linked to an oligonucleotide of Claim 1, 2 or 3;

-98-

1

- b) binding said oligonucleotide to a target mRNA present in said cell type; and
- c) thereby delivering said drug to said specific cell type.

58. A method of detecting a target nucleic acid which comprises:

10 contacting a circular oligonucleotide of any one of Claims 1 to 3 with a sample to be tested for containing said nucleic acid for a time and under conditions sufficient to form an oligonucleotide-target complex; and

detecting said complex.

15 59. The method of Claim 58 wherein said nucleic acid comprises a double-stranded nucleic acid target, and wherein said conditions are effective to denature said target by strand displacement and thereby permit binding of said oligonucleotide to form said oligonucleotide-target complex.

20 60. The method of Claim 58 wherein said sample comprises a pure or impure nucleic acid sample, a tissue section, a cell smear or a chromosomal squash.

61. The method of Claim 58 wherein said conditions effective to denature said target comprise having said circular oligonucleotide and said target present in a ratio of about 1 to about 100.

62. The method of Claim 58 wherein said time effective to denature said target ranges from about 1 minute to about 16 hours.

30

35

-99-

1

63. The method of Claim 58 wherein said complex is detected by a fluorescence energy transfer assay.

5

64. The oligonucleotide of Claim 12 wherein sufficient complementarity is at least about 50%.

10

15

20

25

30

35

FIG. IA 1 / 8

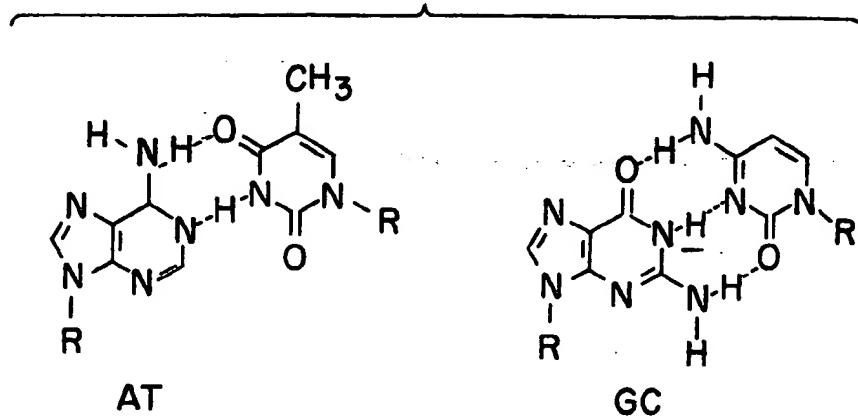
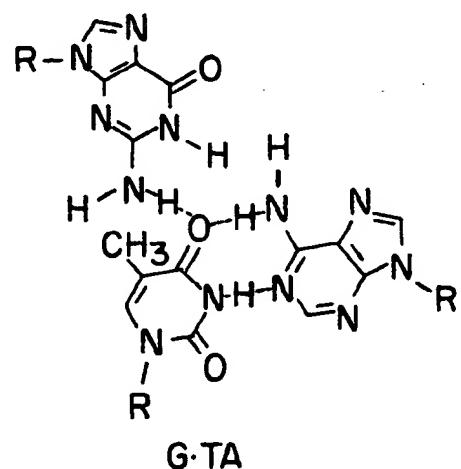
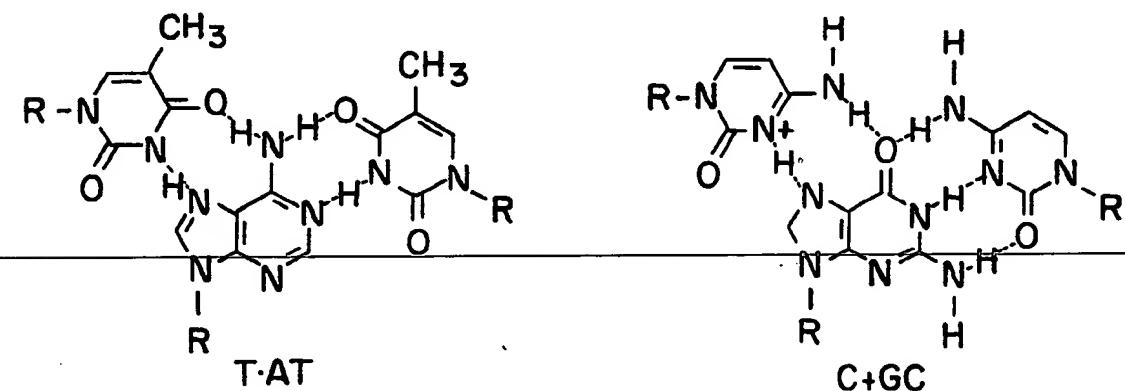
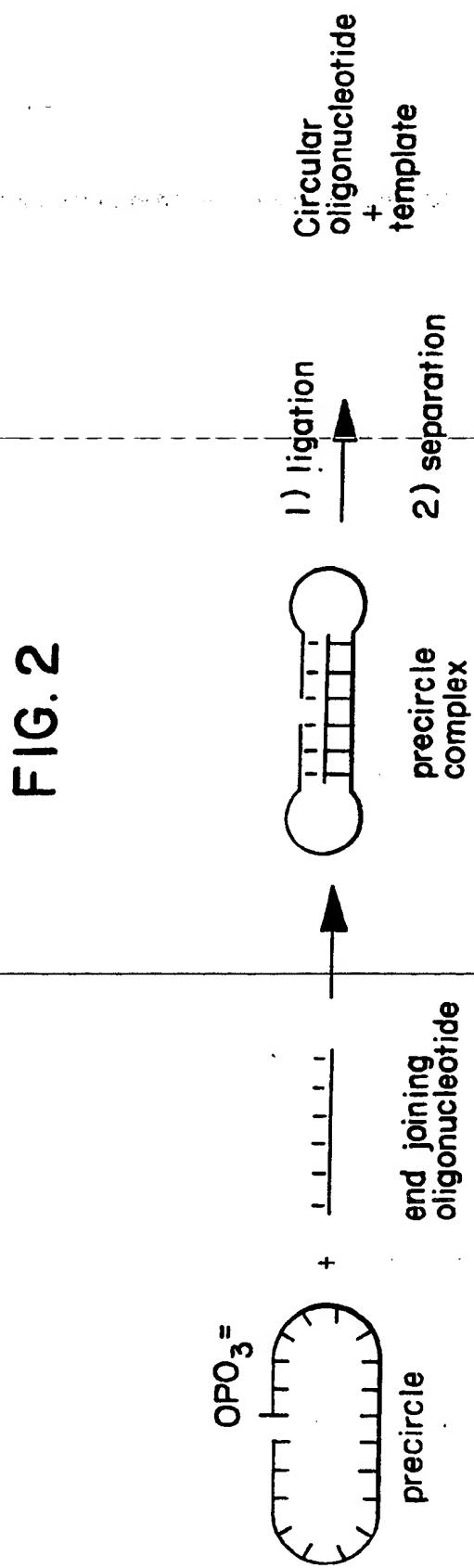


FIG. IB



2 / 8



3 / 8

FIG.3

Precircles (1-3), Targets (4-5), Linear Oligonucleotides (9) and Circles (6-8) used in Experiments

1 5'-TTTTTTCACACTTTTTTTTACACACTTTTT (SEQ ID NO: 5)

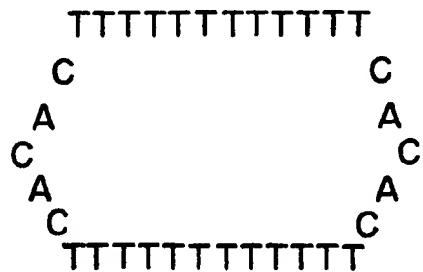
2 5'-TCCTTCCACACCTTCTTCTTCTCACACCTCTT (SEQ ID NO: 6)

3 5'-TTTCTTCACACTCTTTCTTCCACACCTTCT (SEQ ID NO: 7)

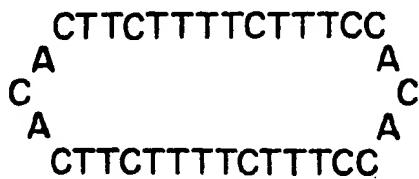
4 5'-AAAAAAAAAAAAA (SEQ ID NO: 8)

5 5'-AAGAAAAGAAAG (SEQ ID NO: 9)

6 | (SEQ ID NO: 5)



7 | (SEQ ID NO: 6)



8 CTTCTTTCTTTCC (SEQ ID NO: 7)



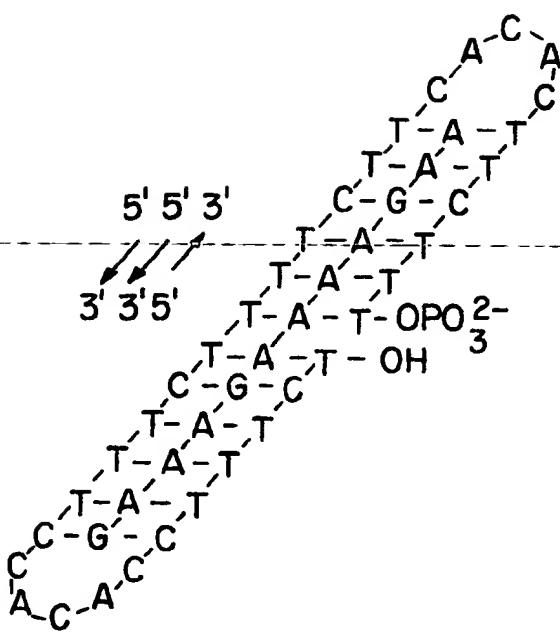
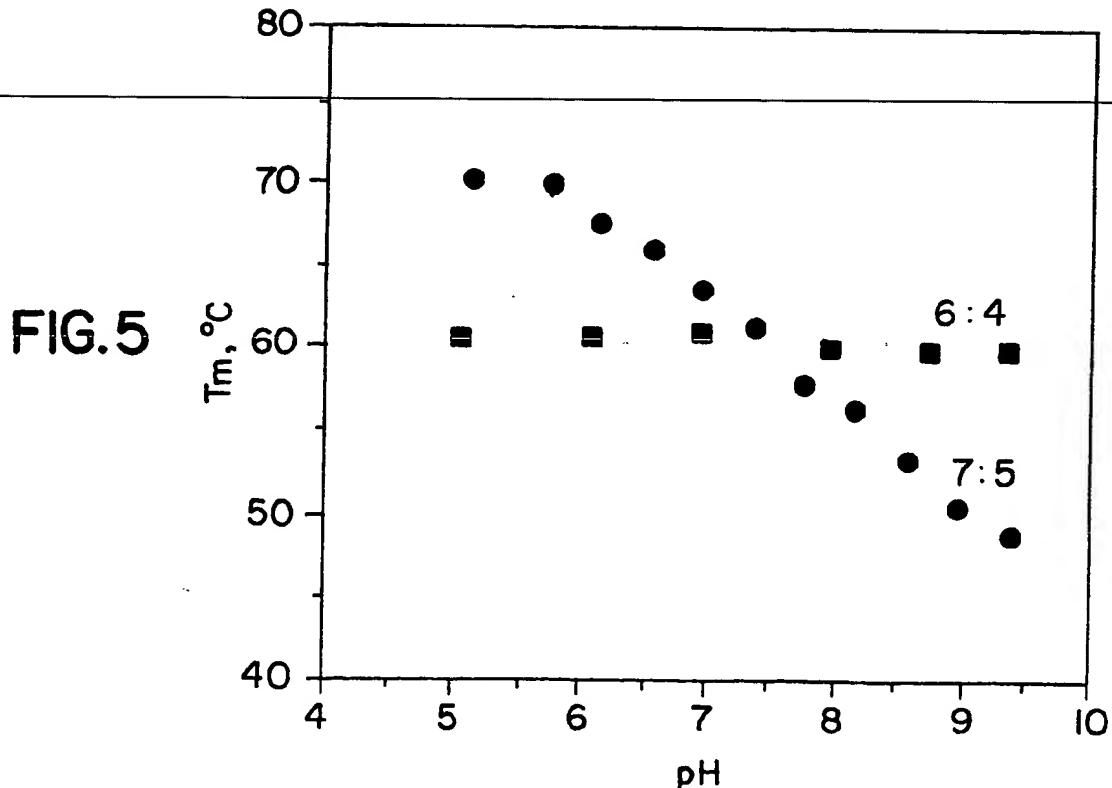
4 / 8

FIG. 3
CONT.

9	5'- CTTTCTTTCTT	(SEQ ID NO: 10)
10	5'- AAGAATAGAAAG	(SEQ ID NO: 11)
11	5'- AAGAAUAGAAAG	(SEQ ID NO: 12)
12	5'- CTTTCTATTCTT	(SEQ ID NO: 13)
13	I TTCTTCTCTTTC C C A A C C A A C C TTCTTATCTTTC	(SEQ ID NO: 14)
14	5'- AAAAAAAAAAAAAA 3'- TTTTTTTTTTTT	(SEQ ID NO: 15)

5 / 8

FIG. 4

pH Effects on T_m

6 / 8

FIG. 6B

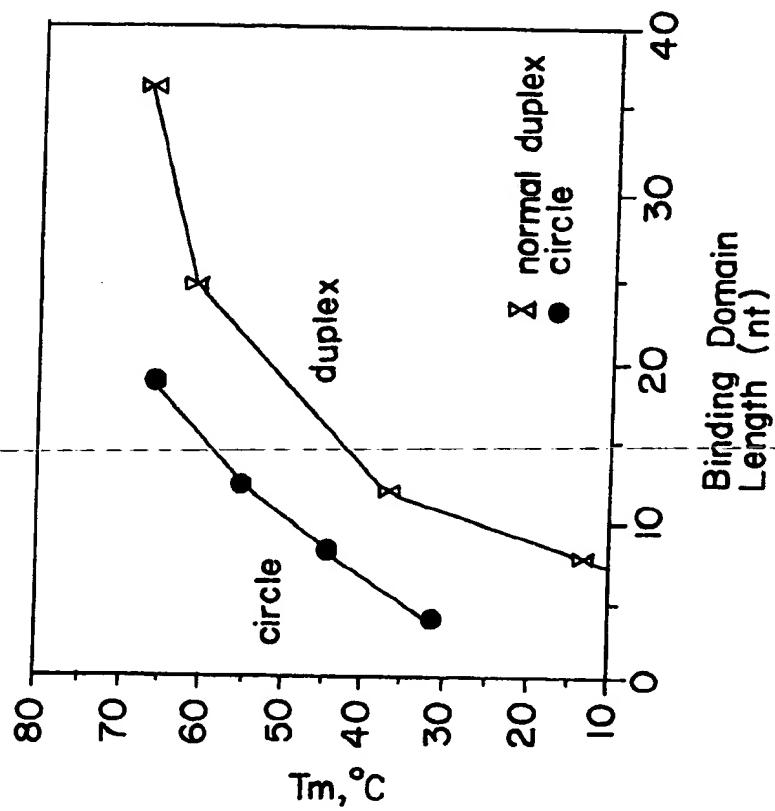
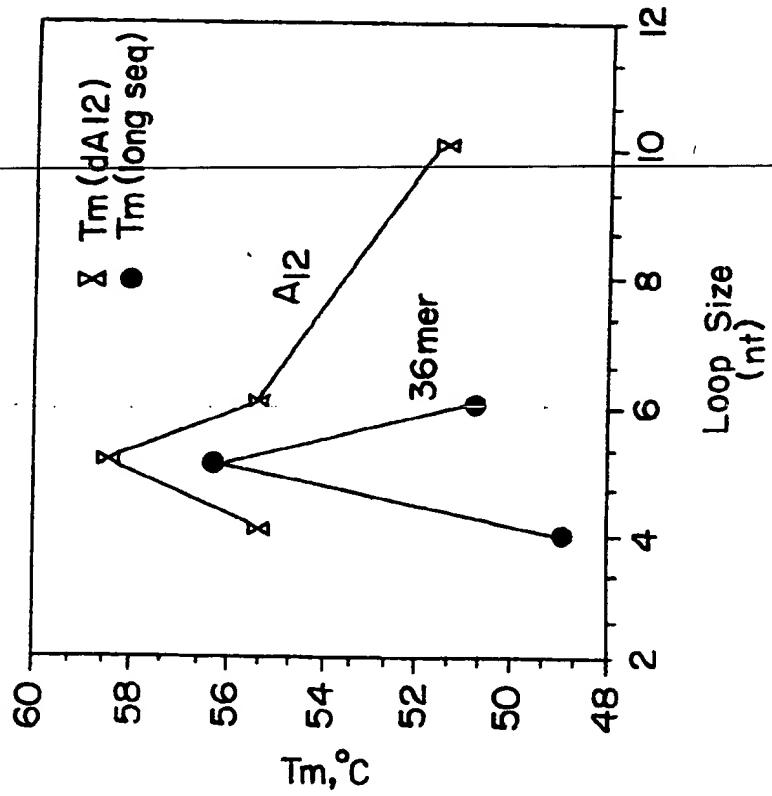
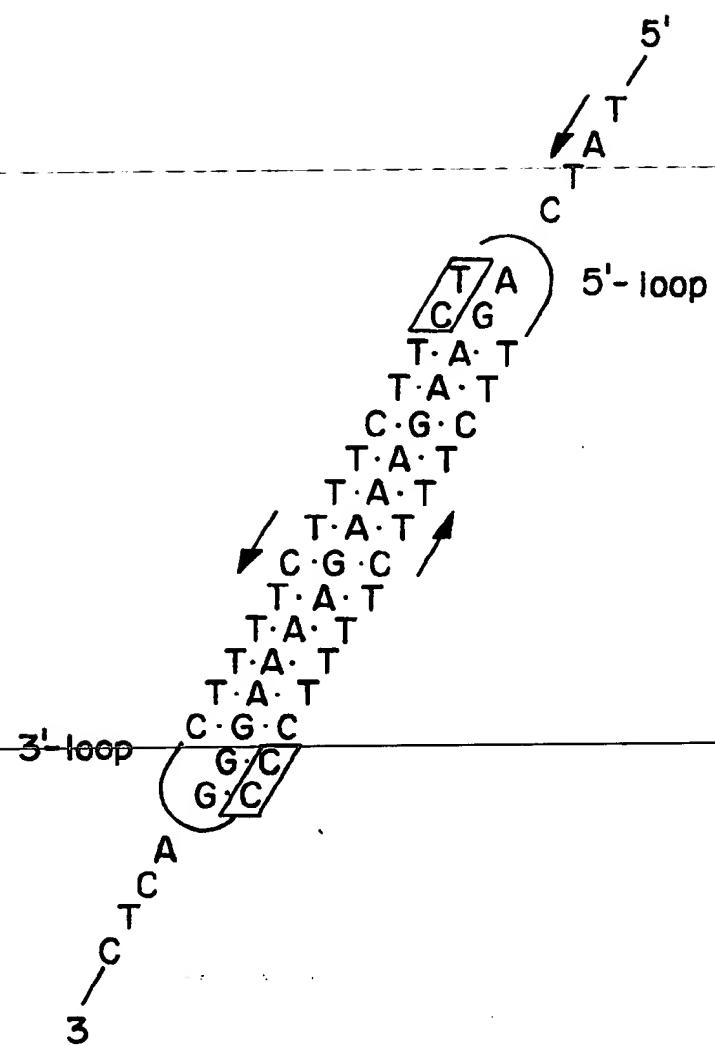


FIG. 6A



7 / 8

FIG. 7



8 / 8

FIG. 8

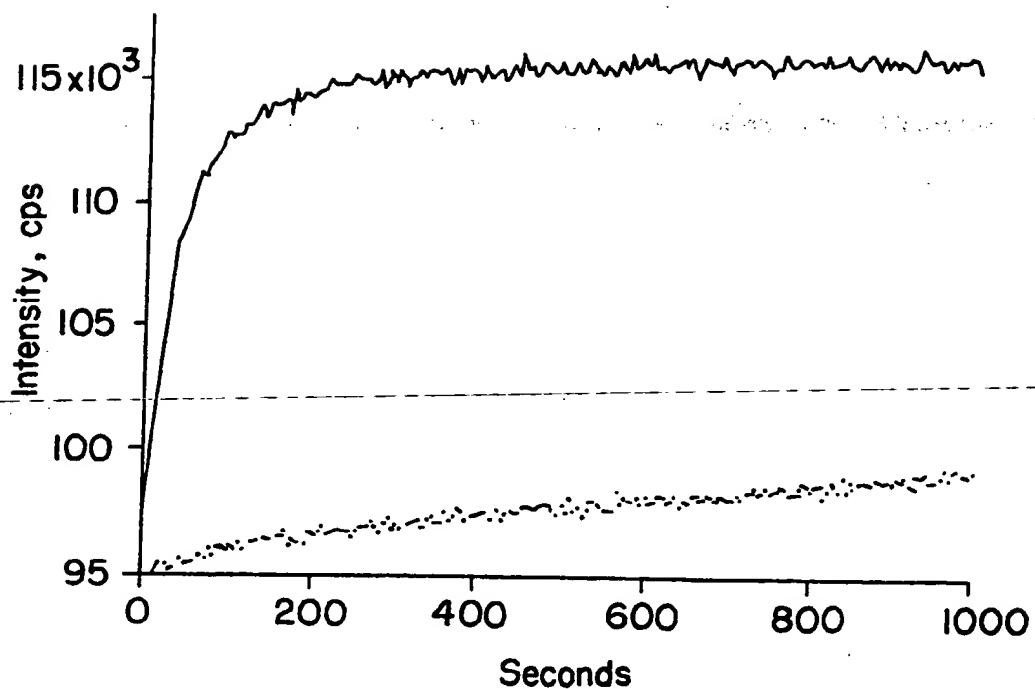


FIG. 9

